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The goal of this study is to determine whether dose-intensive adjuvant regimens for breast cancer induce genetic damage to hematopoietic stem cells, defined by the emergence of clonal hematopoiesis. Two different assays are used to detect clonality: the HUMARA (human androgen receptor) assay to estimate the incidence of early genetic damage defined by the presence of clonal hematopoiesis and microsatellite instability testing to screen for loss of heterozygosity or the presence of defective DNA mismatch repair mechanisms. Study accomplishments to date include: a) SWOG protocol (S9719) activation, b) centralized specimen collection/ processing repository developed, c) clonality assays developed/standardized, d) specimen collection/data analysis of 68 samples from 14 patients completed, e) DOD submission of protocol amendments to increase patient accrual by incorporating S9719 into the clinical treatment (S9623) protocol, and f) presentation of biannual S9719 protocol updates at SWOG group meetings. Analysis of additional patients with longer follow-up is essential to confirm these preliminary results; however, at this point, neither regimen used in this setting (dose-intensive therapy with growth factor support vs. high-dose therapy with stem cell reinfusion for stage II/III breast cancer) appears to initiate genetic damage that could result in development of hematologic malignancies.

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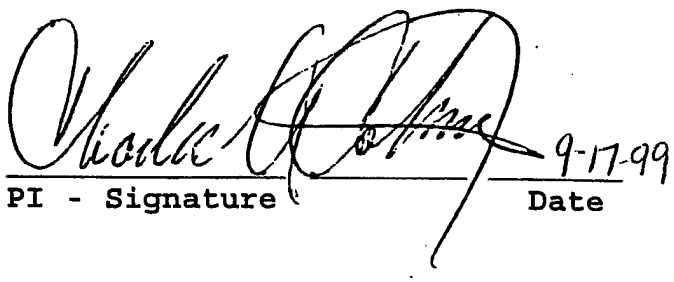

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Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence

ANNUAL REPORT TABLE OF CONTENTS

SOUTHWEST ONCOLOGY GROUP
DAMD17-97-1-7088 ANNUAL REPORT

Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence

INTRODUCTION

Subject: Clonal Hematopoiesis As A Marker Of Genetic Damage Following Adjuvant Chemotherapy For Breast Cancer: Pilot Study To Evaluate Incidence. A Southwest Oncology Group (SWOG) Study.

Purpose and Scope of the Research: The goal of this study is to determine whether dose-intensive adjuvant regimens for breast cancer induce genetic damage to hematopoietic stem cells, defined by the emergence of clonal hematopoiesis. To answer this question, we are studying sequential blood/bone marrow samples from 200 women enrolled in a single, randomized dose-intensive Southwest Oncology Group adjuvant breast cancer study for women with four to nine positive nodes (S9623, "A Comparison of Intensive Sequential Chemotherapy using Doxorubicin plus Paclitaxel plus Cyclophosphamide with High Dose Chemotherapy and Autologous Hematopoietic Progenitor Cell Support for Primary Breast Cancer in Women with 4-9 Involved Axillary Lymph Nodes, Phase III, Intergroup"). Two different assays are used to detect clonality: the HUMARA (human androgen receptor) assay to estimate the incidence of early genetic damage defined by the presence of clonal hematopoiesis and microsatellite instability testing to screen for loss of heterozygosity or the presence of defective DNA mismatch repair mechanisms. In cases where either the HUMARA or microsatellite repeat assay is positive for clonality, the incidence of *MLL* fusion gene transcripts and *RAS* gene mutations (*H-, K-,* and *N-RAS)* will be performed.

BODY

Statement Of Work Objectives/Problems

The Southwest Oncology Group clonal hematopoiesis protocol (S9719) was activated on October 15, 1997. Official notification to all Southwest Oncology Group member institutions occurred in November 1997, with introductory protocol presentations by Drs. Slovak and Stock at the 1997-1998 Southwest Oncology Group group meetings. Despite the advertisements and protocol presentations, patient accrual was slower than anticipated, causing concern for the inability to draw meaningful conclusions within the proposed timeframe. In October 1998, Drs. Slovak and Stock conducted a telephone survey of medical professionals to explore the reasons for suboptimal patient accrual. Results of the survey indicated two major barriers. First, many institutions had not opened the study and were not offering it to their S9623 patients. Secondly, requiring two separate consent forms was cumbersome for the medical professionals and their patients.

To increase patient accrual and obtain the biological information needed to answer our hypothesis, Dr. Slovak sent a letter (dated 10/26/98) to Dr. Charles A. Coltman, Jr. requesting incorporation of the proposed genetic studies (S9719) directly into the primary treatment protocol (S9623). This request was submitted to the Southwest Oncology Group Executive Scientific Committee, the Breast Disease Executive Committee, and the Principal Study Coordinator of S9623, Dr. Scott I. Bearman, for consideration and approval. After an unanimous decision to move forward with this project, an amendment was written to include the following changes: 1) the Southwest Oncology Group will require concurrent registration to S9719 at the time of S9623 registration for Southwest Oncology Group institutions, making S9719 a mandatory ancillary, and 2) the Southwest Oncology Group would remove the model informed consent from the S9719 protocol and incorporate it into the S9623 model informed

consent form. These changes will ensure that the study is opened at all Southwest Oncology Group institutions who register patients to S9623 and that it is offered to patients at the time of S9623 registration. Patients will always have the option to participate in the clinical protocol without the requirement of participating in the biological study. The S9623 study calendar will list the four time points for S9719 specimen collection. Furthermore, with the consent documents combined, medical professional and patients will have only one form to consider at the time of registration. We are optimistic that these changes will boost accrual and allow us to complete the study.

The draft amendments for both protocols were submitted to C. Susan Rupprecht, Contract Specialist US Army Medical Research Acquisition Activity, and Catherine Smith, Department of Defense, Human Use Review Specialist, on June 15, 1999 for DoD review and approval. A copy of the protocol amendments may be found in the appendix (Exhibit 1). Because S9623 is an intergroup clinical protocol, i.e., opened for accrual among three adult cooperative groups, whereas the S9719 clonal hematopoiesis is a 200 patient pilot study limited to Southwest Oncology Group institutions, the timing window to accrue patients is finite. A quick response from the DoD was requested (within two weeks), however, Ms. Karen Stotler informed us the earliest response from the DoD would occur around September 8, 1999 (see appendix, Exhibit 2). We received "unofficial" conditional approval from HSRRB on September 9, 1999 (appendix, e-mail from Louise Pascal, Exhibit 3). After receipt of final approval from Col. Zadinsky, the amendments must be forwarded to CTEP (Cancer Therapy and Evaluation Program) for approval and, finally, to the individual IRBs at each Southwest Oncology Group institution for reevaluation. **As a consequence, since October 1, 1998, the Southwest Oncology Group has not dispersed any year 2 DoD funds to the testing sites to support the research objectives of this grant.** Once the protocol amendments are in place and patient accrual/specimen collection resumes, funding will be distributed to the testing centers. In addition, Southwest Oncology Group institutions have been notified that amendments for S9623 and S9719, as described in the October 1999 Southwest Oncology Group Report of Studies, are pending DoD and CTEP approval (Exhibit 4).

The original Statement of Work did not take into consideration time needed for protocol amendments and the approval process. There are numerous levels of approval required by the Southwest Oncology Group, the NCI, and the DoD, with concomitant lag times between request for consideration, draft review, receipt of feedback, resubmission, and final approval. The Southwest Oncology Group gave this protocol priority status, and every effort was made to facilitate the approval process. Unfortunately, the protocol amendments were only unofficially "conditionally approved" by DoD pending minor adjustments after a three month investigation. We, therefore, remain in a holding pattern until final DoD approval before submission for CTEP approval and local IRB reevaluation at Southwest Oncology Group institutions. This process has been very time consuming, despite being given priority handling. For these reasons, we would like to extend patient registration for an additional 1-2 years to ensure both an adequate study population and receipt of the last sample (12 months after the completion of all therapy). This study has received positive feedback whenever it has been presented, and both clinicians and patients are strongly enthusiastic about the potential contribution of this project to successful treatment outcomes for breast cancer. For these reasons, we are confident the project will be completed as proposed, with a modified timetable. No additional funding will be requested. As indicated above, funding has been placed "on hold" pending approval of the amendments and increased patient accrual.

Collection of specimens has begun and preliminary data analyses of S9719 samples are presented below. Age-matched controls do not appear to be necessary, with the use of T lymphocyte internal controls to normalize skewed X-inactivation patterns.

Experimental Methods/Results to Date

Sample collection: Peripheral blood samples are collected from each patient enrolled on studied at the time points listed in Table 1.

TABLE 1. Time points for Sample Collection for Study of Clonal Hematopoiesis (Arm A – intensive chemotherapy; Arm B – autotransplant)

Time points		Time points	Sample Source
A.	Pretreatment	Arms A + B (200 Women)	Bone Marrow and/or Blood
B.	Stem Cell Collection	Arm B only (100 Women)	Apheresis (peripheral blood stem cells) or Bone Marrow & blood
C.	3 Months Following Completion of All Chemotherapy	Arms A + B (200 Women)	Blood
D.	12 Months Following Completion of All Chemotherapy	Arms A + B (200 Women)	Blood
E.	In case of diagnosis of Secondary Malignancy	Arms A + B	Bone Marrow Blood

*If the patient is also registered to S9702, pretreatment and stem cell collection samples may be collected for that study at the same time.

Two ml of a pretreatment bone marrow aspirate and/or 40 ml of peripheral blood from each patient (required for entry to S9623) are collected in EDTA tubes. These samples serve as a sensitive control to detect any pre-treatment clonality abnormality. Forty ml of blood is also collected at three and twelve months following completion of all chemotherapy. For the 100 women in our study randomized to the autologous stem cell transplant arm of S9623, 2 ml from the stem cell collection is also obtained for analysis. All samples are sent at room temperature by overnight courier to the Southwest Oncology Group tissue repository at the University of New Mexico, directed by Cheryl L. Willman, M.D, where specimens are separated by Ficoll-Paque (Amershanm Pharmacia Biotech, Piscataway, NJ) into their mononuclear and granulocytic layers and frozen separately in liquid nitrogen. Samples are subsequently batched and sent to the City of Hope National Medical Center for nucleic acid isolation. The repository at the University of New Mexico retains frozen cells on all samples for future studies. Cells from each blood sample (a minimum of 20-30 x 10^6 cells) are frozen for viability according to standard methods.

Despite the accrual concerns listed above, 14 patients have been registered to S9719 (clonal hematopoiesis protocol) from ten independent Southwest Oncology Group institutions. These institutions include Columbia River CCOP (n=3), Oregon Health Sciences University (n=2), St Francis/Stormont/Univ. Kansas (n=2), Univ. Arizona (n=1), Harris Methodist/San Antonio, U of TX (n=1), Henry Ford Hospital (n=1), N Colorado Medical Center (n=1), Northwest CCOP (n=1), Salem Hospital (n=1) and Loyola University (n=1). Thirteen of the 14 peripheral blood or bone marrow pretreatment samples were considered adequate for further evaluation. One sample had an inadequate granulocytic (PMN) cell yield. In two patients, only a pretreatment peripheral blood was submitted (minimum requirement for study). Including follow-up samples, a total of 68 samples from 14 patients have been submitted and analyzed.

Sample Processing: In year 2, frozen aliquots of mononuclear cells and granulocytes from all samples were sent by overnight service to Dr. Slovak's Laboratory for nucleic acid isolation, as previously described (1, 2). Briefly, samples were subsequently thawed and washed. An aliquot of 5 x 10^6 cells was frozen for RNA studies using RNA STAT 60 (Tel-Test, Inc., Friendswood, TX). T-cell separation was performed with antibody-labeled magnetic Dynabeads M-450, CD2 (Dynal, Inc. Lake Success, NY). Flow cytometry studies verified granulocyte contamination is less than 1% in the T cell enriched fraction. High molecular weight DNA was prepared using standard proteinase K digestion and phenol/chloroform extraction methods (1). The DNA is split equally for HUMARA (Dr. Marilyn Slovak) and microsatellite

instability (Dr. Wendy Stock) testing. RNA is shipped to Dr. Stock for *MLL* RT-PCR and *RAS* mutation studies (scheduled to be performed in year three for all cases exhibiting clonality).

HUMARA assay: The HUMARA assay is a nested PCR-based test to detect clonality utilizing the human androgen receptor locus on the X chromosome. The assay is highly informative, due to a vastly polymorphic CAG repeat (20 different alleles) in the first exon of the locus, and the ability to quantitate allelic ratios between the active and inactive X chromosomes (3, 4). The HUMARA assay is performed as described by Mach-Pascual *et al.* (5) and quantitated by the method of Delabesse *et al.* (6). Our step-by-step protocol is listed in the appendix (Exhibit 5). DNA is digested with *Rsa I* and *Hpa II* (Roche Diagnostic, Indianapolis, IN) restriction enzymes. The assay amplifies a ~250 to 300 base pair (bp) region of the first exon of the human androgen receptor. Two *Hpa II* methylation sensitive sites reside within 100 bp 5' to the polymorphic CAG repeat. Primers flank the methylation sensitive restriction enzyme sites and the CAG repeat simultaneously. Methylated enzyme (*Hpa II*) sites correlate with X inactivation. Unmethylated alleles (active X) are digested by *Hpa II* and eliminated from PCR amplification. The methylated or inactive allele remains intact after the *Hpa II* digestion and is the only allele amplified. After amplification, maternal and paternal alleles are resolved using a sequencing gel. Random inactivation will show both maternal and paternal alleles, signifying a polyclonal state; a clonal population will be identified by the presence of only one allele or a shift of greater than 3-fold, to control for skewed X-inactivation over the other allele. The 5' primer is labeled with fluorscein, with quantitation of alleles performed using Fluor-imager and ImageQuant software. Samples are run in duplicate and the entire assay is repeated twice to ensure reproducibility. In our preliminary analyses, differences between the maternal (mat) and paternal (pat) alleles were observed in all S9719 patients.

In year one, HUMARA validation studies were completed in collaboration with D. Gary Gilliland, M.D., Ph.D., Brigham and Women's Hospital, Harvard Medical School, Boston, MA, to address quantitative, interlaboratory variability, quality control and reproducibility issues. In year two, the HUMARA assay was performed on 68 samples from 14 S9719 patients. All 14 patients were informative and showed polyclonal hematopoiesis in their pretreatment sample. In addition, all follow-up samples were within normal limits. Figure 1 shows the results of three S9719 samples. Each sample comprises eight lanes; the first four lanes of each sample contain T cell DNA digested with *Rsa I* alone (lanes 1, 2, 9, 10, 17, 18) or *Rsa I* and *Hpa II* (lanes 3, 4, 11, 12, 19, 20). *Rsa I*, a restriction enzyme that is not sensitive to DNA methylation status, cuts the DNA outside the trinucleotide repeat of the human androgen receptor gene locus, allowing for visualization of both mat and pat alleles based on their differences in CAG repeat number. Lanes 1 and 2 of each sample (T cell or PMN) are digested with *Rsa I* only and show both mat and pat alleles. Lanes 3 and 4 have been digested with both *Rsa I* and *Hpa II*, which determines inactive allele status. In Figure 1, samples 165292, 164192, and 166407 exhibit polyclonal hematopoiesis of a corrected ratio 1.0 to 1.1 [corrected ratio for both allele amplification and T-cell tissue-specific (internal control)], whereas the PMN positive control (lanes 25-28) exhibits a skewed X-inactivation ratio of 4.0 mimicking clonal hematopoiesis. Positive or skewed samples have a ratio of $\geq$ 3:1.

Two correction factors are necessary for this assay. The allele ratio is defined as the ratio between the two chromosome X-linked alleles in a given sample. The corrected ratio, which corrects for preferential amplification of one of the two alleles, is determined by dividing *Rsa* I-*Hpa* II by *Rsa* I alone. Those samples with a corrected ratio of less than 3 are considered within normal limits. Those with a corrected ratio greater than 3 are consistent with either skewed X inactivation or clonal hematopoiesis. Although criteria for nonrandom X-inactivation are arbitrary, an allele ratio $\geq$ 3:1, which corresponds to the expression of 75% of one allele, has been widely accepted in the literature (5, 7-11).

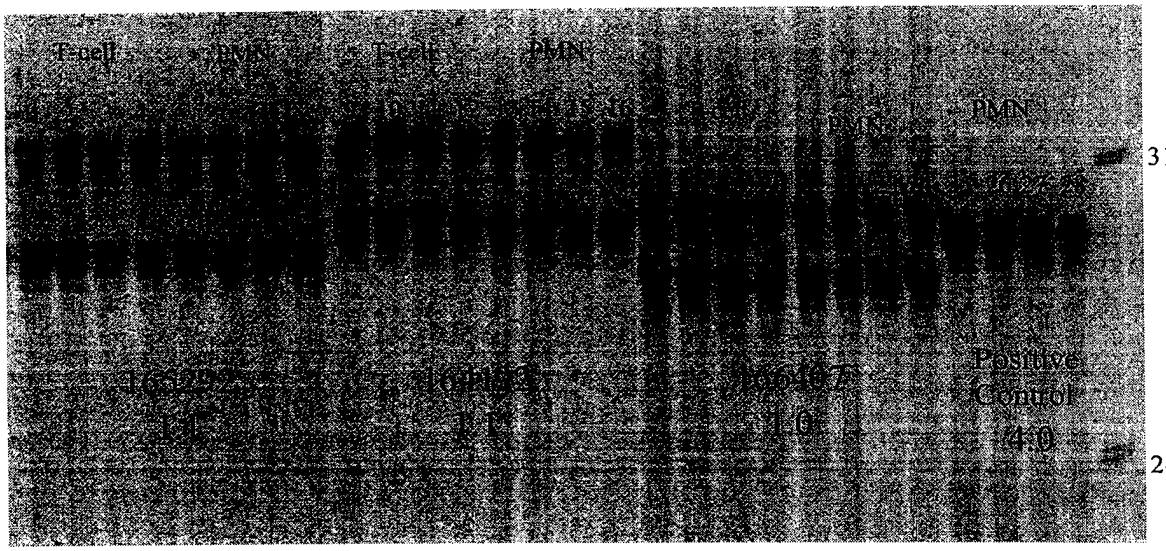


Figure 1 HUMARA gel with three S9719 pretreatment samples. Each sample comprises eight lanes; the first four lanes of each sample contain T cell DNA digested with *Rsa I* alone (lanes 1, 2, 9, 10, 17, 18) or *Rsa I* and *Hpa II* (lanes 3, 4, 11, 12, 19, 20). The last four lanes of each sample contains polynuclear mononuclear (PMN) DNA digested with *Rsa I* alone (lanes 5, 6, 13, 14, 21, 22, 25, 26) or *Rsa I* and *Hpa II* (lanes 7, 8, 15, 16, 23, 24, 27, 28). The corrected allele ratio (corrects for preferential amplification of one of the two alleles) is determined by dividing *Rsa* I-*Hpa* II by *Rsa* I alone. Those samples with a corrected ratio of less than 3 are considered within normal limits. All three S9719 patients exhibit polyclonal hematopoiesis. The positive control (lanes 25-28) shows a skewed allelic ratio of 4.0. Positive or skewed samples have a ratio of ≥ 3:1.

In addition, our study has a second correction that internally controls for skewed X-inactivation or excessive Lyonization. This refers to a skewed X-inactivation that occurs in females who have randomly inactivated a preponderance of one X-chromosome (either paternal or maternal). Gale and colleagues (10) have estimated this occurs in ~23% of females and has been reported to increase with age, with >30% of the normal population having skewed X inactivation patterns (XIP) at age 60 (9). Because XIP may vary from tissue to tissue, somatic controls from embryologically related tissues are needed to determine and interpret skewed X inactivation patterns (10). The use of T cells, as the control tissue, eliminates false positives due to skewed X-inactivation that mimic a clonal population and controls for age related skewing. Previous studies that indicated clonality studies using an X-inactivation based assay should incorporate age-matched controls did not collect T-cells for an internal control to eliminate false positive results due to skewed X-inactivation. In our study, the ratio of the two alleles in the experimental tissue (polymorphonuclear cells) must be divided by the ratio of the same two alleles in normal somatic control tissue (T-cells). As above, if the ratio remains greater than 3, the results are consistent with clonal hematopoiesis. Figure 2 provides an example of skewed X-inactivation normalized with T lymphocytes.

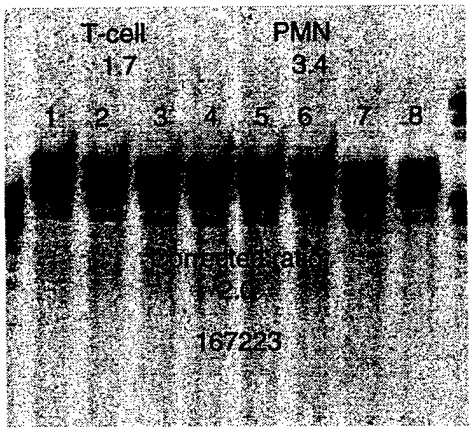
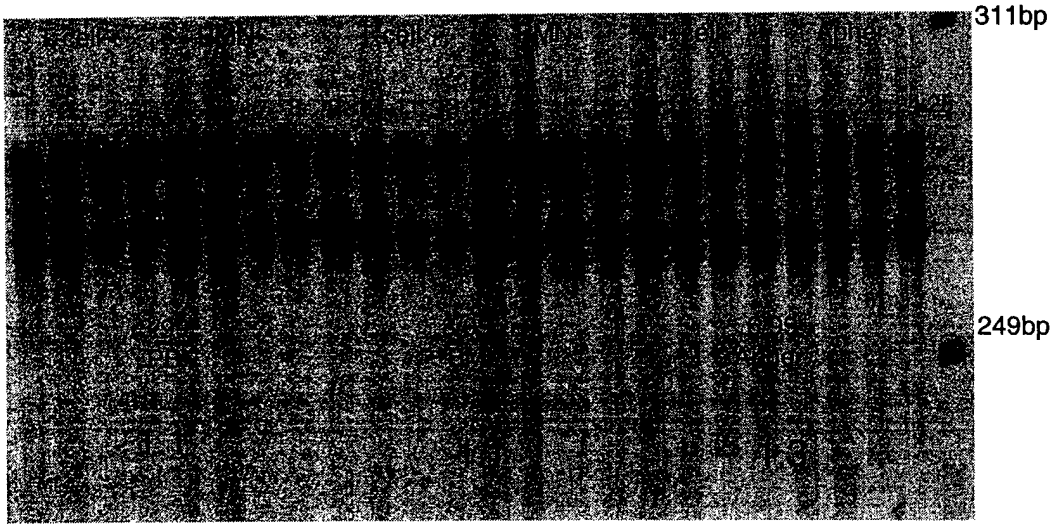


Figure 2 Example of skewed X-inactivation in an S9719 patient. T-cell *Rsa I* ratio is 1.1 and the T-cell *RsaI/HpaII* ratio is 1.9. The PCR correction for allele amplification in T cell DNA is 1.9/1.1 or 1.7. PMN Rsa *I* ratio is 1.1 and the PMN *RsaI/HpaII* ratio is 3.7. The PCR correction for allele amplification in PMN is 3.7/1.1 or 3.4 (skewed X-inactivation in PMN). Tissue specific correction ratio is defined as PMN corrected ratio/T-cell corrected ratio or 3.4/1.7 or 2.0 (within normal limits).

Although our test population is limited, to date only one of the 14 patients studied in this prospective study has evidence of age-related skewing or excessive Lyonization. This skewing was normalized using T lymphocytes as a tissue specific internal control. In a related prospective study investigating patients being treated with high dose chemotherapy with or without stem cell rescue for lymphoma, leukemia, or multiple myeloma, T-cell corrected ratios provide correction for skewed X-inactivation or excessive Lyonization. Previously, others (9) and we encountered problems associated with skewed inactivation, due to our an inability to obtain pretreatment (baseline) samples or failure to obtain T lymphocytes for patient and tissue-specific internal controls. Accordingly, the need for age-matched female controls is very important in retrospective analyses, where T lymphocyte controls are not readily available.

The HUMARA assay has been found to be reliable and reproducible in studies of human neoplasia (4, 5, 8, 12-15). In a retrospective analysis of 104 female patients from a single institution undergoing autologous bone marrow transplantation (ABMT) for NHL, polyclonal hematopoiesis was identified in 77%, a skewed chromosome X-inactivation pattern (unable to be corrected due to a lack of T lymphocyte controls) was observed in 20%, and 3% or 3 patients had clonal hematopoiesis at the time of BMT (5). To further test the predictive value of clonality for the development of t-MDS/AML, a subgroup of 78 patients with at least 18 months of follow-up, was evaluated. Clonal hematopoiesis predicted the development of t-MDS/AML in 3 of 4 patients, approximately 7 to 15 months before clinical diagnosis of disease.

In our prospective study, seven of 14 patients have pretreatment, apheresis product (for patients on transplant arm), and a limited number of follow-up samples. For five patients, follow-up times are too early to test. Presentation peripheral blood and bone marrow samples with a follow-up apheresis sample collected three months later, from patient #167145 are presented in Figure 3. All samples show polyclonal hematopoiesis with a corrected ratio range of 1.1 to 1.3.



Patient # 167145

Figure 3 Sequential samples from patient #167145. This HUMARA gel illustrates three samples from one S9719 patient: peripheral blood (lanes 1-8) and bone marrow (lanes 9-16) (both collected at disease presentation) and the apheresis sample (lanes 17-24) collected 3 months later. Each sample comprises eight lanes. Lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, and 22, are RSA I digestion only; lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, 24 are digested with *Rsa* I and *Hpa II.* Lane 25, DNA size markers. The patient demonstrates polyclonal hematopoiesis with a corrected ratio range of 1:1.1 to 1.3.

Microsatellite Instability Assays: Microsatellite instability (MSI) assays have been chosen as an adjunct method to the HUMARA for detection of a clonal hematopoietic stem cell population that emerges as a result of chemotherapy-induced genetic damage. Since receiving funding from the DoD, we have identified 10 MSI markers and have developed protocols for the amplification and detection of these markers in experimental tissue (granulocytic fraction of blood or mononuclear fraction of bone marrow) and control (buccal mucosa or peripheral blood T cell) populations (16-20). The first 5 MSI markers were chosen on the basis of existing literature documenting their utility in the detection of MSI in a variety of different malignancies. These markers include: BAT26, BAT40, APC, Mfd15CA, and D2S123. The other five markers are also highly informative and were chosen on the basis of their location in genomic regions where chromosome translocations or loss of heterozygosity (LOH) have been reported frequently in therapy-related leukemias and, specifically, where abnormalities have been associated with topoisomerase II inhibitors. These MSI markers (with genomic location) include: AFM240YA11 (3q21), AFM302xb9 (11q23), AFM031xc5 (21q22), AFM337zg5 (12p12) and AFMb298yh5 (20pter-20qter).

As of September, 9, 1999, we have completed MSI analysis using BAT26, BAT40, APC, Mfd15CA, and D2S123 markers on all patients registered to the protocol to date (See appendix for MSI figures, Exhibit 6). This includes a total of 14 patients; in seven of these patients, pretreatment, apheresis product (for patients on transplant arm), and limited numbers of follow-up samples have been analyzed. In the remaining seven patients, only pretreatment blood and marrow analyses have been completed; no follow-up samples have yet been received. To date, no microsatellite instability has been detected. All assays have been highly reproducible.

Our strategy remains to screen for MSI using the initial five markers. If MSI is detected at any of these loci, we plan to examine MSI with the second panel of five microsatellite markers that are located in genomic regions frequently involved in leukemia-specific translocations and/or deletions. Similarly, RT-

PCR assays for several of the frequently reported 11q23 fusion transcripts in t-MDS/AML [t(9;11), t(11;19)] will be performed in any case where clonality is detected by either HUMARA or MSI.

Clearly, additional patients and more long-term follow-up is required to confirm these findings; however, to date, there is no evidence to suggest that genetic damage, as assayed by MSI, occurs as a result of either form of therapy in the Southwest Oncology Group intergroup study for women with stage II/III breast cancer.

Microsatellite Instability Analysis:

Microsatellite Markers:

BAT26

5'-TGA CTA CTT TTG ACT TCA GCC-3'
5'-AAC CAT TCA ACA TTT TTA ACC C-3'

Location: 2p

PCR conditions:	annealing temp.: 55°C
$MgCl_2$ concentration:	2.5mM
Primer amount/reaction:	5pmol
Template amount/rxn:	100ng
Amplicon size:	~80-100bp

BAT40

5'-ATT AAC TTC CTA CAC CAC AAC-3'
5'-GTA GAG CAA GAC CAC CTT G-3'

Location: 1p13.1

PCR conditions:	annealing temp.: 58°C
$MgCl_2$ concentration:	1.5mM
Primer amount/reaction:	5pmol
Template amount/rxn:	100ng
Amplicon size:	~80-100bp

APC

5'-ACT CAC TCT AGT GAT AAA TCG-3'
5'-AGC AGA TAA GAC AGT ATT ACT AGT T-3'

Location: 5q21/22

PCR conditions:	annealing temp.: 58°C
$MgCl_2$ concentration:	3.5mM
Primer amount/reaction:	5pmol
Template amount/rxn.:	100ng
Amplicon size:	96-122bp

Mfd15CA 5'-GGA AGA ATC AAA TAG ACA AT-3'
5'-GCT GGC CAT ATA TAT ATT TAA ACC-3'

Location: 17q11.2-q12

PCR conditions:	annealing temp.: 52°C
$MgCl_2$ concentration:	2.5mM
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon size:	~150bp

AFM093xh3 (D2S123) 5'-AAA CAG GAT GCC TGC CTT TA-3'
5'-GGA CTT TCC ACC TAT GGG AC-3'

Location: 2p16

PCR conditions:	annealing temp.: 60°C
$MgCl_2$ concentration:	2.5mM
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	197-227bp

AFM240YA11 (D3S1309) 5'-CTT TGG GGA ATC ATT AGT CTG T-3'
5'-ATG AGA ATT GTC ATG GTG C-3'

Location: 3q21-q25.2

PCR conditions:	annealing temp.: 56°C
$MgCl_2$ concentration:	1.5mM
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	141bp

AFM302xb9 (D11S1345) 5'-TGC CAC AGT AAT ACA TGT GTG TAA T-3'
5'-TAG TCA GTG CTG AGC CCA TA-3'

Location: 11q23.3-24.2

PCR conditions:	annealing temp.: 55°C
$MgCl_2$ concentration:	
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	235bp

AFM337zg5 5'-GAG GGG TGG CAT CTC T-3'
(D12S363) 5'-CTC AAA TGA AAT CAG CAT AAA-3'

Location: 12p12-p11.2

PCR conditions:	annealing temp.: 55°C
$MgCl_2$ concentration:	
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	208bp

AFMb298yh5 5'-TCA AGA GAT TTA TTG TCC AAC AAG-3'
(D20S887) 5'-ATT GAG TGG GTT CAG ACT CC-3'

Location: 20pter-qter

PCR conditions:	annealing temp.: 55°C
$MgCl_2$ concentration:	
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	245-267bp

AFM031xc5 5'-GAA ATG TTT TAA TAA ATG GTG GTT A-3'
(D21S270) 5'-ACA AAG TTA TGG TCA AGG GG-3'

Location: 21q22.2-21qter

PCR conditions:	annealing temp: 55°C
$MgCl_2$ concentration:	
Primer amount/rxn:	5pmol
Template amount/rxn:	100ng
Amplicon length:	199-223bp

PCR primer pairs:

The forward primer of each primer set contains a 5'-end labeled fluorophore to allow for automated fluorescence detection. One fluorophore (6Fam, Hex, or Tet: blue, yellow, green fluorescence respectively) is end labeled to each forward primer.

PCR reactions:

All PCR reactions contain 100 ng of either normal (T cell) or experimental (PMN) DNA and 2.5 units of Amplitaq Gold polymerase (Perkin-Elmer). The PCR reaction is a "hot start" reaction: The Amplitaq Gold polymerase must be heated initially at 95°C for 12 minutes in order to be activated for amplification.

Parameters: 95°C x 12 min

95°C x 30 sec
x 30 sec
72°C x 30 sec
} 45 cycles

72°C x 10 min

25°C hold

Analysis:

2µl of each PCR product is run on a 4% polyacrylamide denaturing gel:

Each product is combined with a 350-bp size standard (Perkin-Elmer) labeled with Tamra (red fluorescent fluorophore).

The gels are run on an ABI 377 instrument and are analyzed following electrophoresis using Genescan Analysis software. The Genescan software collects raw signals emitted by each fluorophore. Every fragment in a peak contributes a single fluorophore: peak area is directly proportional to the number of molecules. The Genotyper DNA Fragment Analysis Software aids in determining the allele sizes of the amplified products. Therefore, the control and "tumor" allele sizes can be compared for determination of microsatellite instability.

Detection of *RAS* mutations and *MLL* gene rearrangements: In cases where the HUMARA or microsatellite repeat assays are positive for clonal hematopoiesis, sensitive reverse-transcriptase PCR assays, using RNA from banked specimens, will be used to determine whether *RAS* mutations and *MLL* fusion transcripts commonly reported in therapy-related myelodysplasia and AML have occurred. These studies will be performed in the last year of data analysis. To date, all samples analyzed are polyclonal.

Statistical Considerations

One hundred patients per arm from S9623 will be accrued on this study. The length of accrual will be modified to 3-5 years. Compliance with the three-month blood draw is expected to be near 100%; at 12 months following completion of treatment, approximately 15% are anticipated to have relapsed, or refused and not have samples available. The probability of clonal hematopoiesis at a particular timepoint can be established within +/- 0.1 with a sample size of 100 per arm, and to within +/- 0.11 with a sample size of 85. A two-sided .05 level test of the association of the treatment group with presence or absence of clonality will have adequate power to detect differences of .25 or greater (power at least .93 for the pretreatment and three month timepoint and .88 for the 12 month post-treatment timepoint, if sample size decreases to 85 per arm). Association of other pre-study patient characteristics and tumor-related variables, with presence or absence of clonality by HUMARA or microsatellite assays, will also be explored.

KEY RESEARCH ACCOMPLISHMENTS

- 14 patients have been entered onto protocol and samples have been analyzed. In 7 of 14 patients, pretreatment, apheresis (in patients randomized to the transplant arm), and short-term follow-up samples have been analyzed for clonal hematopoiesis using the HUMARA as well as microsatellite instability assays. All 14 patients gave informative results. No evidence of clonal hematopoiesis or microsatellite instability was detected.

- Sequential peripheral blood collected at regular intervals may detect clonal populations resulting from genetic damage following dose-intensive adjuvant regimens for breast cancer.
- The HUMARA and microsatellite instability assays give reproducible and complementary results.
- HUMARA and microsatellite instability assays can be performed reproducibly from sequentially obtained blood samples of women treated on this study.
- Peripheral blood T-lymphocytes are a useful internal, tissue-specific control for the HUMARA assay precluding the need of age-matched controls for skewed X-inactivation.

REPORTABLE OUTCOMES

Analysis of additional patients with longer follow-up is essential to confirm these very preliminary results; however, at this point, neither regimen used in this setting (dose-intensive therapy with growth factor support vs. high-dose therapy with stem cell reinfusion for stage II/III breast cancer) appears to initiate genetic damage that could result in development of secondary hematologic malignancies.

A peripheral blood tissue bank for all samples submitted for this study has been developed at the Southwest Oncology Group tissue repository, University of New Mexico. This bank is directed by Cheryl L. Willman, M.D. The specimens are separated by Ficoll-Paque (Amershanm Pharmacia Biotech, Piscataway, NJ) into their mononuclear and granulocytic layers and frozen separately in liquid nitrogen. The repository at the University of New Mexico retains frozen cells on all samples for future studies. Cells from each blood sample (a minimum of 20-30 x 10^6 cells) are frozen for viability according to standard methods.

CONCLUSIONS AND FUTURE DIRECTIONS

This pilot study is designed to test the hypothesis that genetic damage, defined by the presence of clonal hematopoiesis, can be detected in a subset of patients following dose-intensive adjuvant therapy for breast cancer. Study accomplishments at the end of year 2 include: a) Southwest Oncology Group protocol (S9719) activation, b) centralized specimen collection/processing repository developed, c) clonality assays developed/standardized, d) specimen collection/data analysis of 68 samples from 14 patients completed, e) no evidence of clonal hematopoiesis or microsatellite instability detected, f) presentation of biannual S9719 protocol updates at Southwest Oncology Group group meetings, and g) DoD submission of draft protocol amendments to increase patient accrual by incorporating S9719 into the clinical treatment (S9623) protocol. The protocol is simple and only requests blood to be drawn at four time points for those patients agreeing to participate in the study. We are optimistic that the protocol amendment to combine S9719 with the clinical treatment (S9623) will boost accrual and allow us to complete the study.

The confirmation, that adjuvant chemotherapy can induce clonal hematopoiesis in a significant number of patients, from this pilot study will **provide a unique model to prospectively study the evolution of therapy-related leukemogenesis in patients being treated for breast cancer**, and would be the focus of a subsequent grant proposal. The goals of a larger study would include the following: 1) to determine whether a relationship exists between detection of clonal hematopoiesis and subsequent evolution to t-MDS/AML; 2) to identify general mechanisms (e.g., faulty DNA repair and mutations in components of cell cycle checkpoints, which may predispose patients to genetic instability and leukemogenesis following adjuvant therapy; 3) to determine the sequence of events (genomic instability, loss of heterozygosity, specific mutations/translocations, etc.) which participate in leukemogenesis; and 4) to determine whether specific adjuvant regimens place patients at an unacceptably high risk for the development of therapy-related hematologic malignancies. If the study is negative, high risk breast cancer patients can rest assured that they can receive high dose chemotherapy, without an increased risk of development of

clonal hematopoiesis, or subsequent evolution to a therapy-related hematopoietic disorder, when compared to the general population.

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APPENDIX

Attached are the following appendices.

Exhibit 1: DoD notification and amendment protocols for S9719 and S9623 dated 6/15/99

Exhibit 2: E-mails (dated 7/14/99 to 8/10/99) from Ms. Karen Stotler: Protocol amendment review by HSRRB planned for 9/8/99

Exhibit 3: E-mail from Louise Pascal indicating conditional "unofficial" HSRRB approval on 9/9/99

Exhibit 4: Southwest Oncology Group Report of Studies indicating an amendment to increase patient accrual is in progress, pending DoD and CTEP approval

Exhibit 5: HUMARA assay protocol

Exhibit 6: Microsatellite instability data sheets for two S9719 patients. Comparison of bone marrow, peripheral blood or apheresis product to T lymphocytes (control) (labeled as BML, PBL or T-cell) to the granulocytic layer (BM, PMN, second pheresis) are presented for two patients. Neither patient shows any evidence for MSI.

EXHIBIT 1

Southwest
Oncology Group
A National Clinical Research Group

June 15, 1999

C. Susan Rupprecht, Contract Specialist
US Army Medical Research Acquisition Activity
Attn: MCMR-AAA-B
820 Chandler Street
Fort Detrick, MD 21702-5014

Catherine Smith
Department of Defense
Human Use Review Specialist
Headquarters, USAMRMC
Fr. Detrick, MD 21702-5012

RE: **S9719** (DAMD17-97-1-7088), "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence, Ancillary to **S9623**." Study Coordinators: Drs. M. Slovak, C. Willman, W. Stock, and K. Albain.

Dear Ms. Rupprecht and Ms. Smith:

Patient accrual for the above-noted study has not been rapid enough to ensure its completion within a reasonable timeframe. Through discussion with medical professionals within the Southwest Oncology Group, we have become aware of two possible barriers to accrual. First, many institutions have not opened the study and are not offering it to their **S9623** patients. Secondly, requiring two separate consent form documents is cumbersome for both the medical professionals and their patients.

Thus, we propose making the following changes to **S9719** and **S9623**: 1) We will require concurrent registration to **S9719** at the same time as **S9623** registration for Southwest Oncology Group institutions, making **S9719** a mandatory ancillary (although patients will still be able to opt out of participation on **S9719**). This will ensure that the study is opened at all Southwest Oncology Group institutions who register patients to **S9623**, and that it is offered to the patient at the time of **S9623** registration; 2) We will remove the model informed consent from the **S9719** protocol and incorporate it into the **S9623** model informed consent. With the consent documents combined, medical professionals and patients will have only one form to consider at the time of registration. We are optimistic that these changes will boost accrual and allow us to complete the study.

Attached, please find the draft amendments to both protocols for your consideration. We hope that these changes are acceptable to you. If you have any comments or questions regarding these proposed changes, please forward them to my attention at the Southwest Oncology Group Operations Office no later than June 25, 1999. If I have not heard from you by that date, I will assume that you have no comments and I will forward the amendments to the Cancer Therapy and Evaluation Program for review and approval.

Thank you for your attention to this matter.

Sincerely,

Tamra N. Oner
Protocol Coordinator

PC/tno

cc: Charles A. Coltman, Jr., M.D.
Peter M. Ravdin, Ph.D., M.D.
Silvana Martino, D.O.
Marilyn L. Slovak, Ph.D.
Cheryl L. Willman, M.D.
Wendy Stock, M.D.
Kathy S. Albain, M.D.
Scott I. Bearman, M.D.
Stephanie J. Green, Ph.D.
Danika Lew, M.A.
Dana B. Sparks, M.A.T.
Gina P. Lozano
Marjorie A. Godfrey

Southwest
Oncology Group
A National Clinical Research Group

_____, 1999

TO: ALL SOUTHWEST ONCOLOGY GROUP, CCOP AND CGOP MEDICAL ONCOLOGISTS AND PATHOLOGISTS

FROM: Tamra N. Oner/Protocol Coordinator

RE: **S9719**, "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence, Ancillary to **S9623**." Study Coordinators: Drs. M. Slovak, C. Willman, W. Stock, and K. Albain.

AMENDMENT #3

Study Coordinator: Marilyn L. Slovak, M.D. Phone: 818/359-8111 ext 2348
E-mail: mslovak@smtplink.Coh.ORG

AMENDMENT #3

The above-noted study is now a mandatory ancillary to the Southwest Oncology Group treatment protocol, S9623. This requirement will remain in effect until accrual on S9719 is completed. Specific protocol changes are listed below:

1. Sections 3.1 and 6.1 have been amended to state that patients must be registered to **S9719** at the same time they are registered to **S9623**, within the same registration phone call.

2. Consent for this study has been incorporated into the **S9623** Model Informed Consent and the **S9719** Model Informed Consent has been removed from the protocol.

3. Section 11.0, Appendix, has been repaginated due to removal of the Model Informed Consent.

Replacement pages are attached for pages 5, 10, 15 and 18. Please append this memo to the front of your copy of the protocol and insert the replacement pages. Also, please remove the Model Informed Consent from your copy of the protocol.

This memorandum serves to inform the Southwest Oncology Group Statistical Center, and the NCI.

cc: Stephanie Green, Ph.D.
Danika Lew, M.A
Diana Lowry
Camille White
Sarah Effert
Karin Rantala
Marjorie Godfrey
C. Susan Rupprecht
Catherine Smith

using pretreatment (baseline) samples to control for variables such as age or damage that may have occurred due to other risk factors/exposures. This study will only evaluate high risk patients (no low risk patients). Two different assays (the HUMARA and microsatellite instability) will be used to detect clonal hematopoiesis as a marker of genetic damage in this pilot study. S9623 will compare the clinical outcome produced with autologous peripheral blood progenitor cell (PBPC) supported high-dose therapy with that of intensive, sequential chemotherapy in Stage II/III breast cancer patients involving 4 - 9 axillary lymph nodes.

Inclusion of Women and Minorities:

This study involves only patients previously registered to S9623. No new patients will be registered.

DRAFT

3.0 ELIGIBILITY CRITERIA

Each of the criteria in the following section must be met in order for a patient to be considered eligible for registration. Use the spaces provided to confirm a patient's eligibility. **This section does not need to be submitted to the Statistical Center.**

____ 3.1 Patients with Stage II/III breast cancer involving 4 - 9 axillary lymph nodes enrolled on S9623 will be eligible for this study. **Patients must be registered at the time of registration to S9623, within the same registration phone call.**

____ 3.2 A pretreatment sample of forty (40) ml of peripheral blood (four 10 ml EDTA tubes supplemented with tissue culture medium) or four provided tubes (per Section 4.2) and when available, a 2 - 4 ml aliquot of pretreatment bone marrow aspirate (collected in EDTA tubes supplemented with tissue culture medium or provided tubes) must be collected prior to beginning treatment on S9623.

____ 3.3 All patients must be informed of the investigational nature of this study and must sign and give written informed consent in accordance with institutional and federal guidelines.

____ 3.4 At the time of patient registration, the date of institutional review board approval for this study must be provided to the Statistical Center.

4.0 PROCEDURES/SAMPLE SUBMISSION REQUIREMENTS

4.1 **Study Design.** For this pilot, samples will be obtained prior to initiation of treatment, from collected stem cell specimens (in 100 patients randomized to autologous stem cell transplant arm), and at three and twelve months following completion of treatment. For details of sample time points see Table 1 below.

5.0 STATISTICAL CONSIDERATIONS

One hundred patients per arm from S9623 will be accrued on this study. The length of accrual is anticipated to be 3 years. Compliance with the three month blood draw should be nearly complete; at 12 months following completion of treatment, approximately 15% might be anticipated to have relapsed or refused and not have samples available. The probability of clonal hematopoiesis at a particular time point can be established to within ± 0.1 with a sample size of 100 per arm, and to within ± 0.11 with a sample size of 85. Change in status between pretreatment, stem cell collection, and the three and twelve month post-treatment samples will be explored, as will concordance of the HUMARA and microsatellite assays. Association of treatment, pre-study, patient characteristics, and tumor-related variables with presence or absence of clonality by HUMARA or microsatellite assays will also be explored. For example, a two-sided .05 level test of the association of the treatment group with presence or absence of clonality will have adequate power to detect differences of .25 or greater (power at least .93 for the pretreatment and three month time point and .88 for the 12 month post-treatment time point, if sample size decreased to 85 per arm).

6.0 REGISTRATION GUIDELINES

6.1 Patients must be registered on this protocol **at the same time they are registered to S9623, within the same registration phone call.**

6.2 At the time of registration, the caller must be prepared to answer every question on the S9719 Registration Form.

6.3 The caller must also be prepared to provide the date of institutional review board approval for this study. Patients will not be registered if the IRB approval date is not provided or is > 1 year prior to the date of registration.

7.0 DATA SUBMISSION SCHEDULE

7.1 Data must be submitted according to the protocol requirements for **ALL** patients registered, including patients deemed to be ineligible. Patients for whom documentation is inadequate to determine eligibility will generally be deemed ineligible.

7.2 Master forms are included in Section 10.0 and (with the exception of the sample consent form) must be photocopied for data submission to the Southwest Oncology Group Statistical Center.

7.3 Members and CCOPs must submit one copy of all data forms directly to the Statistical Center in Seattle. CGOPs must submit (number of copies to be determined by the Group Member) copies of all forms to their Group Member institution for forwarding to the Statistical Center.

7.4 AT REGISTRATION:

Submit pre-study bone marrow (when available) and blood per Sections 4.2a and 4.3 to the University of New Mexico.

10.0 MASTER FORMS SET

This section includes copies of all data forms which must be completed for this study. These include:

10.1 Model Consent Form (deleted as of _____)

10.2 S9719 Registration Form

10.3 Specimen Submission Form

11.0 APPENDIX

ASSAY DESCRIPTIONS

11.1 Sample Processing:

High molecular weight DNA will be prepared from the blood and apheresis samples following Ficoll-gradient separation, according to standard proteinase K digestion and phenol/choroform extraction methods. *(27)* Cells from each blood samples (a minimum of 20 - 30 x10^6 cells) will be frozen for variability according to standard methods. *(28)*

HUMARA assay: DNA samples from each of the 200 patients enrolled will be studied at the time points outlined previously. Clonality at the HUMARA locus will be assessed by PCR amplification according to Willman et al. using the primers described by Gale et al., and quantitated by the method of Delabesse et al. *(21, 29, 30)* Willman et al have performed mixing experiments which demonstrate that the percentage of clonal cells can be estimated with an error of ± 10%, and that a clonal population of cells can be detected if they constitute more than 10 percent of the cells in a polyclonal background. *(21)* Assays will performed in duplicate or triplicate.

Microsatellite instability assay: Microsatellite instability will be assessed at multiple chromosomal loci: 7q31 (D7S522 marker), 5q31 (Mfd27 marker), 17p12 (Mfd41 marker), 8p22 (LPL marker), 11q23(D11S939 marker) and the BAT loci (25, 26 and 40). *(31 - 37)* Although the microsatellite assay is a general assay for genomic instability, we have chosen highly polymorphic microsatellites from regions known to be associated with t-MDS/AML since these markers may also provide information about loss of heterozygosity in these genomic regions. The PCR assays will be done in duplicate according to published methods. *(25 - 26)*

Detection of MLL gene rearrangements and *RAS* mutations: In cases where the HUMARA or microsatellite repeat assays are positive for clonal hematopoiesis, sensitive reverse-transcriptase PCR assays, using RNA from banked specimens, will be used to detect MLL fusion transcripts commonly reported in AML with 11q23 abnormalities. *(38 - 39)* *RAS* mutations will be performed according to published methods. *(4, 15)*

Southwest
Oncology Group
A National Clinical Research Group

DRAFT

_____, 1999

TO: ALL SOUTHWEST ONCOLOGY GROUP, CCOP AND CGOP MEDICAL ONCOLOGISTS, SURGEONS, RADIATION ONCOLOGISTS AND PATHOLOGISTS; CALGB, ECOG, NCCTG AND MDACC

FROM: Tamra N. Oner, Protocol Coordinator

RE: **S9623**, "A Comparison of Intensive Sequential Chemotherapy using Doxorubicin plus Paclitaxel plus Cyclophosphamide with High Dose Chemotherapy and Autologous Hematopoietic Progenitor Cell Support for Primary Breast Cancer in Women with 4-9 Involved Axillary Lymph Nodes, Phase III, Intergroup." Study Coordinators: Drs. S. Bearman, W. Peters and L. Pierce.

AMENDMENT #8

Study Coordinator: Scott I. Bearman, M.D. Phone: 303/372-9000
E-mail: sbearman@entente.uhcolorado.edu

AMENDMENT #8

The above-noted study has been amended to require that all patients registered to this study by Southwest Oncology Group institutions also be registered concurrently to the ancillary study S9719, "Clonal Hematopoiesis as a Marker of Genetic damage Following Adjuvant Chemotherapy for Breast Cancer; Pilot Study to Evaluate Incidence", within the same registration phone call. This requirement will remain in effect until accrual on S9719 is completed. This requirement does not apply to other cooperative group participants on the study.

Additionally, the timing of pulmonary function tests, MUGA and ECG requirements prior to registration have been modified to allow up to 84 days prior to registration for these tests.

The specific protocol changes are listed below:

1. A new eligibility requirement requiring concurrent registration to **S9719** at the time of patient registration to **S9623** has been added as a new Section 5.10. The rest of Section 5.0 has been renumbered accordingly.

2. A new question has been added to the Eligibility Checklist as item #18 asking, "Will the patient be registered to the companion protocol, **S9719**, immediately following the **S9623** registration AND during the same registration phone call? Indicate 'N/A' if the patient is being registered by a non-Southwest Oncology Group institutuion. For Southwest Oncology Group Institutions: Indicate 'N/A' if the patient refuses consent for **S9719** - this is the only exception allowed and must be documented on the initial **S9623** flow sheet."

3. A new bolded sentence has been added after the first paragraph of Section 13.1 stating that, "**Southwest Oncology Group institutions must register patients to the companion protocol, S9719, at the same time as the S9623 registration, within the same registration phone call. (If the patient refuses to give consent for S9719, the refusal must be documented on the initial S9623 flow sheet.)**"

4. A new Section 15.7 has been added to Special Instructions, Section 15.0, specifying that Southwest Oncology Group institutions must enroll patients on the mandatory ancillary study, **S9719**, at the time of **S9623** registration, within the same registration phone call. If the patient refuses to give consent to **S9719**, the refusal must be documented on the initial **S9623** flow sheet.

5. A new Section XII has been added to the Model Informed Consent as signature block for providing **S9719** consent. Also, the specifics of the **S9719** Model Informed consent have been added to the **S9623** Model Informed Consent as specified below:

   a. Information regarding **S9719** has been added to Section I as a new paragraph #6.

   b. A statement has been added as the second sentence in the second to the last paragraph (paragraph #7) of Section I specifying that if patients participate in **S9719**, samples of blood (and possibly marrow) may be preserved for research and development purposes, and that by signing the **S9719** signature block portion of the consent, patients are authorizing the preservation and use of the blood (and marrow) samples taken from them.

   c. A new paragraph has been added as the third to the last paragraph of the end of Section II providing information about the blood and apheresis samples which will be submitted for patients participating on **S9719**.

   d. A new second paragraph has been added to Section IV for patients participating in **S9719**. It specifies that there may be other ways of determining genetic damage comparable to the methods used in **S9719**. It also states that patients have the option of not having the procedure done on their blood (and marrow) samples.

   e. A second paragraph has been added to Section VII for patients participating in **S9719**, stating that their records may be made available to the National Cancer Institute, the Food and Drug Administration, the Southwest Oncology Group and the U.S. Army Medical Research and Material Command.

6. Sections of 5.6d - f have been revised to allow pulmonary function tests, ECG and MUGA within "84 days prior to registration" rather than "between definitive surgery and registration."

7. The face page has been updated to include email addresses for the Southwest Oncology Group study coordinators and statisticians. Also, the ECOG study chair information has been updated on page 2; Dr. Wolff replaces Dr. Holland.

8. The word "calculated" has been added before "creatinine clearance" on the Study Calendars, Sections 9.1 - 9.3.

Replacement pages are attached for the Eligibility Checklist, face page and pages 2, 28, 46 - 48, 50, 56a, 66 - 67a and 76 - 77a. Pages 67a and 77a have been added to prevent extensive repagination. Please append this notice to the front of your copy of the protocol and insert the replacement pages.

This memorandum serves to inform the Southwest Oncology Group Statistical Center, ECOG, CALGB, NCCTG, MDACC and the NCI.

cc:
Stephanie J. Green, Ph.D.
Danika Lew, M.A.
Sarah Effert
Diana Lowry
Karin Rantala
Camille White
Jean MacDonald - ECOG
Kathleen Karas - CALGB
Janis Gjervik - NCCTG
Debbie Frye - MDACC
Rae Aldridge - MDACC
Brian Koziol - Amgen
Marjorie A. Godfrey
C. Susan Rupprecht
Catherine Smith

S9623 Eligibility Checklist:

The Eligibility Checklist for S9623 is under revision and will be added to the protocol prior to CTEP submission of the amendment.

PRIVILEGED COMMUNICATION
FOR INVESTIGATIONAL USE ONLY
Amended 11/1/96

Revised 3/15/97
Amended 6/1/97

S9623
Activated July 1, 1996
Amended _____

SOUTHWEST ONCOLOGY GROUP

A COMPARISON OF INTENSIVE SEQUENTIAL CHEMOTHERAPY USING DOXORUBICIN PLUS PACLITAXEL PLUS CYCLOPHOSPHAMIDE WITH HIGH DOSE CHEMOTHERAPY AND AUTOLOGOUS HEMATOPOIETIC PROGENITOR CELL SUPPORT FOR PRIMARY BREAST CANCER IN WOMEN WITH 4 - 9 INVOLVED AXILLARY LYMPH NODES

PHASE III (INTERGROUP)

PARTICIPANTS: ALL SOUTHWEST ONCOLOGY GROUP, CCOP AND CGOP MEDICAL ONCOLOGISTS, SURGEONS, RADIATION ONCOLOGISTS AND PATHOLOGISTS; CALGB, ECOG, NCCTG AND MDACC

STUDY COORDINATORS:

Scott I. Bearman, M.D. (BMT)
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AGENTS:

BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea) (NSC-409962) (Carmustine)(BiCNU)
Carboplatin (NSC-241240)(CBDCA)
Ciprofloxacin hydrochloride
Cisplatin (CDDP) (Platinol®) (NSC-119875)
Cyclophosphamide (Cytoxan®) (NSC-26271)
Doxorubicin (NSC-123127)
Filgrastim (r-metHuG-CSF) (NSC-614629)
Paclitaxel, Taxol® (NSC-673089)
Tamoxifen (Nolvadex) (NSC-180973)
Thiotepa (NSC-6396)

Lori J. Pierce, M.D. (Radiation Oncology)
Department of Radiation Oncology
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STUDY COORDINATORS (CALGB S9623):

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STUDY COORDINATORS (NCCTG):

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Rochester, MN 55905

STUDY COORDINATORS (MDACC):

Zia Rahman, M.D.
Department of Breast Medical Oncology
Box 56
1515 Holcombe Boulevard
Houston, TX 77030

Please contact the Southwest Oncology Group Study Coordinator (Dr. Bearman) for questions relating to treatment.

b. CBC (WBC ≥ 3,000/µl, ANC ≥ 1,000/µl, platelets ≥ 100,000/µl) within 28 days prior to registration.

c. 24 hour urine for creatine clearance or calculated creatinine clearance by the formula below (must be ≥ 60 ml/minute) within 28 days prior to registration.

$$\text{Estimated creatinine clearance} = \frac{(140 - \text{age}) \times \text{wt (kg)} \times 0.85}{72 \times \text{creatinine (mg/dl)}}$$

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d. Pulmonary function tests showing FVC, FEV1, DLCO all ≥ 60% of predicted within 84 days prior to registration.

e. ECG within 84 days prior to registration. In the event of abnormal ECG, candidates must be cleared by a cardiologist.

f. Resting MUGA with left ventricular ejection fraction ≥ 45% within 84 days prior to registration.

g. No uncontrolled or significant cardiovascular disease; no congestive heart failure; no serious cardiac conduction abnormalities such as second or third degree heart block; no atrial or ventricular arrhythmias. Patients who are on medication known to affect cardiac conduction are eligible if they receive these medications for reasons other than heart failure or arrhythmia and are cleared by a cardiologist.

h. Patients must be HIV negative. Hepatitis B surface antigen and hepatitis C status must be known. Hepatitis and HIV testing must be performed within 42 days prior to registration.

5.7 Mammogram of the opposite breast must be performed within 16 weeks of registration. Results must be one of the following: normal, abnormal but abnormalities determined not to be breast cancer, or synchronous breast cancer. Patients who have undergone bilateral mastectomies are not required to undergo mammography of the contralateral breast.

5.8 No other prior malignancy is allowed except for the following: adequately treated basal cell or squamous cell skin cancer, in situ cervical cancer, intraductal or lobular carcinoma *in* situ of the breast (diagnosed at any time), or any other cancer from which the patient has been disease-free for 5 years. Bilateral breast cancer patients are not eligible except for synchronous (diagnosed at the same time, i.e., within four weeks of initial histologic diagnosis) carcinoma of the contralateral breast, provided neither tumor is N3 or T4, both sides have < 10 nodes involved, one or both have 4 - 9 nodes involved, and either a modified radical mastectomy or breast sparing surgery plus axillary node dissection (meeting the criteria in Section 5.2) has been performed for both tumors.

5.9 Patients must not have serious medical or psychiatric illness which prevents informed consent or participation in this trial.

5.10 **If the patient is being registered by a Southwest Oncology Group institution**, the patient must also be registered to the companion hematopoiesis protocol, **S9719**, at the time of registration to **S9623**, within the same registration phone call, unless the patient refuses to give consent for **S9719**. (See **S9719** for more information.)

5.11 All patients must have been evaluated and approved for this study by a transplant center approved for this study by one of the participating cooperative groups (see Section 19.4). This evaluation includes confirmation of bed availability at the transplant center and confirmation that a written request for insurance approval has been submitted (and/or confirmation of ability to pay for transplant). This evaluation must take place prior to registration.

5.12 Pregnant or nursing women may not participate. Men are ineligible. Women of child-bearing potential must be planning to use effective contraception.

5.13 All patients must be informed of the investigational nature of this study and give written informed consent in accordance with institution and federal guidelines.

5.14 At the time of registration, the date of institutional review board approval for this study must be provided to the Statistical Center.

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9.0 9.1 STUDY CALENDAR -- ARM 1 (Sequential intensive chemotherapy)

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		D1	D8	D15	D22	D29	D36	D43	D50	D57	D64	D71	D78	D85	D92	D99	D106	D113	D120	D155
REQUIRED STUDIES	PRE	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk
	STUDY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	23
PHYSICAL ¥																				
History and Physical	X							X						X						
Pelvic Examination	X*																			
Weight & Performance Status	X							X						X						
Toxicity Notation		X		X		X		X		X		X		X		X		X		
LABORATORY																				
CBC/Differential/Platelets ¥ π	X	X		X		X		X		X		X		X		X		X		
Bilateral Bone Marrow biopsies	X																			
40 cc blood for S9719 Ø	X																			
Measured/calculated creatinine clearance	X																			
Hepatitis/HIV	X																			
Total bilirubin	X	X		X		X														
Serum sodium ∞														X∞		X∞		X∞		
SGOT	X																			
Urinalysis Δ	X																			
Pregnancy test Σ	X																			
PFTs/DLCO	X																			
ER/PgR Assay	X																			
Pathology submission	X																			
X-RAYS AND SCANS																				
Mammogram Ω	X																			
Chest X-ray ®	X																			
Bone Scan	X																			
CT chest/abd/pelvis	X#																			
MUGA	X													X¶						
ECG	X																			
TREATMENT ß																				
Doxorubicin		X		X		X														
Paclitaxel								X		X		X								
Cyclophosphamide														X		X		X		
G-CSF *f*		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Ciprofloxacin *f*		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Tamoxifen £																				
Radiotherapy √																				X

NOTE: Forms are found in Section 18.0. Forms submission guidelines may be found in Section 14.0.

¥ After completing chemotherapy, repeat physician visit with blood studies every four months for three years, then every six months for two years, then annually. A pelvic examination is required annually.

* A pelvic examination within six months prior to registration is required prestudy. A pelvic examination is required annually. Women who have had their uterus completely removed are exempt from the requirement for prestudy and annual pelvic examinations.

π CBC, differential and platelets must be performed immediately prior to each cycle of chemotherapy.

∞ Serum sodium should be evaluated if there is a change in mental status within 24 hours of cyclophosphamide administration (see Section 8.1c.4).

Δ As clinically indicated.

Ω Repeat annually for opposite breast as well as for affected breast in patients who received breast-conserving surgery. Not applicable for patients who have had bilateral mastectomies.

® Repeat annually for 5 years, then as indicated.

Repeat as clinically indicated.

¶ Must be performed prior to the first cycle of cyclophosphamide.

f To be given on Days 3-10, 17-24, 31-38, 45-52, 59-66, 73-80, 87-94, 101-108, 115-122. Note: If ciprofloxacin is not available, another quinolone antibiotic may be substitued. Please see Section 7.1.a for substitution instructions.

£ Begin four weeks after completion of the last course of adjuvant chemotherapy; 20 mg po qd for five years for postmenopausal patients and hormone receptor-positive premenopausal patients only.

√ Patients who have had a breast sparing procedure and axillary dissection must begin external beam RT 4-6 weeks after adjuvant chemo. is completed.

ß All doses are based on adjusted ideal body weight (see Section 7.1).

Σ Pregnancy test required for all women of reproductive potential.

Ø For patients on Arm 1 who are also registered to S9719, a pre-treatment peripheral blood sample of 40 ml is required per S9719 protocol. Two to four ml of bone marrow (when available) should also be submitted concurrently. Forty ml of peripheral blood is also required for submission (per S9719 protocol) at 3 and 12 months following completion of all chemotherapy and in the event a secondary malignancy is diagnosed.

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9.0 9.2 STUDY CALENDAR -- ARM 2 (Induction chemotherapy followed by HDCT)

		Day 1	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43	Day 50	Day 57	Day 64	Day 71	Day 78	Day 85
REQUIRED STUDIES	PRE	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk	Wk
	STUDY	1	2	3	4	5	6	7	8	9	10	11	12	13
PHYSICAL ¥														
History and Physical	X				X			X			X			X
Pelvic Examination	X*													
Weight & Performance Status	X				X			X			X			X
Toxicity Notation					X			X			X			X
LABORATORY														
CBC/Differential/Platelets ¥ π	X		X	X	X	X	X	X	X	X	X	X	X	X
Bilateral Bone Marrow biopsies	X													
40 cc blood for S9719 Ø	X													
Measured/calculated creatinine clearance	X													
Hepatitis/HIV	X													
Total bilirubin	X				X			X			X			X
SGOT	X													
Urinalysis Δ	X													
Pregnancy test Σ	X													
PFTs/DLCO	X													
ER/PgR Assay	X													
Pathology submission	X													
X-RAYS AND SCANS														
Mammogram ß	X													
Chest X-ray	X													
Bone Scan	X													
CT chest/abd/pelvis	X#													
MUGA	X													
ECG	X													
TREATMENT														
Doxorubicin		X			X			X			X			
Cyclophosphamide		X			X			X			X			

NOTE: Forms are found in Section 18.0. Forms submission guidelines may be found in Section 14.0.

* A pelvic examination within six months prior to registration is required prestudy. Women who have had their uterus completely removed are exempt from the requirement for prestudy pelvic examinations.

π CBC, differential and platelets must be performed weekly starting Day 8 during therapy.

Δ As clinically indicated.

\# Repeat as clinically indicated.

ß Mammogram not applicable for patients who have had bilateral mastectomies.

Σ Pregnancy test required for all women of reproductive potential.

Ø For patients on Arm 2 who are also registered to S9719, a 40 ml pre-treatment blood sample is required for submission per S9719 protocol. Two to four ml of bone marrow (when available) should also be submitted concurrently.

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9.0 9.3 STUDY CALENDAR -- ARM 2 (High-dose chemo with Autologous Progenitor Cell Support)

REQUIRED STUDIES	PRE	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day
		*													¥ Ø
	HDCT	-7	-6	-5	-4	-3	-2	-1	0	7	14	21	28	42	180
PHYSICAL ¥															
History and Physical	X														
Weight & Performance Status	X														
Toxicity Notation ¶		X	X	X	X	X	X	X		X	X	X	X		
LABORATORY															
CBC/Differential/Platelets ¥ π	X		X	X	X	X	X	X	X						
40 cc blood for S9719 Ø	X														
2-4 ml apheresis specimen for S9719 Ø															
Measured/calculated creatinine clearance	X														
Total bilirubin	X														
SGOT	X														
PFTs/DLCO	X														
X-RAYS AND SCANS															
Mammogram Ω															
Chest X-ray ®	X														X
Bone Scan															X
CT chest/abd/pelvis #															X
MUGA	X*f*														
ECG	X	X	X	X	X	X									
TREATMENT															
AHPC Collection ∞ Ø	X														
AHPC Reinfusion §									X						
G-CSF or GM-CSF Σ									X	X	X				
STAMP I															
Cyclophosphamide			X	X	X										
Cisplatin			X	X	X										
BCNU						X									
STAMP V															
Cyclophosphamide		X	X	X	X										
Carboplatin		X	X	X	X										
Thiotepa		X	X	X	X										
Tamoxifen £														X	
Radiotherapy √														X	

NOTE: Forms are found in Section 18.0. Forms submission guidelines may be found in Section 14.0.

¥ After completing chemotherapy, repeat physician visit with blood studies every four months for three years, then every six months for two years, then annually. A pelvic examination is required annually (except for women who have had their uterus completely removed).

* High-dose chemotherapy begins no more than six weeks following the last dose of induction chemotherapy (see Section 9.2). Contact Dr. Bearman if a longer delay is anticipated.

Repeat as clinically indicated.

¶ Toxicity evaluation should be performed daily during HDCT and then weekly until discharge.

π CBC, differential and platelets must be performed daily until discharge to home.

Ω Repeat annually for opposite breast as well as for affected breast in patients who received breast-conserving surgery. Not applicable for patients who have had bilateral mastectomies.

® Repeat annually for 5 years, then as indicated.

f MUGA must be ≥ 45% prior to the start of high dose chemotherapy.

∞ AHPC collection may include bone marrow and/or peripheral blood progenitor cells.

§ AHPC reinfusion should begin on Day 0. Reinfusion may take place over more than one day.

Σ G-CSF or GM-CSF should be started on the day of the first reinfusion of AHPC and should continue until granulocyte engraftment according to institutional guidelines.

£ Begin four weeks after completion of the last course of adjuvant chemotherapy; 20 mg po qd for five years for postmenopausal patients and hormone receptor-positive premenopausal patients only.

√ Patients who have had a breast sparing procedure and axillary dissection must begin external beam RT after adjuvant chemo. is completed.

Ø For patients on Arm 2 who are also registered to S9719, 40 ml peripheral blood and 2 - 4 ml of apheresis specimen must be submitted at stem cell collection (per S9719 protocol). Forty ml of peripheral blood is also required at 3 and 12 months following completion of all chemotherapy and in the event a secondary malignancy is diagnosed.

11.4 Data Monitoring Committee (DMC)

This study will be monitored throughout accrual and follow-up periods by the Southwest Oncology Group Data Monitoring Committee (DMC). There is a single DMC to monitor all Southwest Oncology Group Phase III therapeutic trials. This committee is responsible for reviewing interim analyses prepared by the study statistician and for recommending whether the study needs to be modified or terminated based on these analyses. This committee also determines when the study results will be submitted for publication or otherwise released to the public. It will review any major modifications to the study proposed by the Study Committee.

The Study Committee consists of the Study Coordinators, study Statistician, Discipline Coordinators and a representative from each participating Group for intergroup studies. The Study Committee is responsible for monitoring the data from the study for toxicity, feasibility and accrual. The study committee also initiates minor changes in the study such as clarification of eligibility criteria.

12.0 DISCIPLINE REVIEW

Discipline review is not required.

13.0 REGISTRATION GUIDELINES

13.1 Registration, Southwest Oncology Group Investigators: All patients must be registered with the Southwest Oncology Group Statistical Center by telephoning 206/667-4623, 6:30 a.m. to 5:00 p.m. Pacific time, Monday through Friday, excluding holidays. Patients must be registered prior to initiation of treatment no more than one working day prior to the planned start of treatment. **No exceptions will be permitted.**

NOTE: ALL Southwest Oncology Group institutions must register patients to the companion protocol, S9719, at the same time as S9623 registration, within the same registration phone call, unless patients refuse to give consent for S9719. (If the patient refuses to give consent for S9719, this refusal must be documented on the initial S9623 flow sheet.)

Registration, NCCTG Investigators: All patients will be registered by calling 507/284-4130, or Faxing a completed Eligibility Checklist (507/284-0885) to the NCCTG Randomization Center between 8:30 a.m. to 4:30 p.m., Central Time, Monday through Friday, excluding holidays. The NCCTG Randomization Center will obtain and confirm all eligibility criteria. The NCCTG office will then contact the Southwest Oncology Group Statistical Center to randomize the patient and will contact the institution to relay the treatment assignment. A confirmation of randomization will be forwarded to the institution through the NCCTG office. Patients must be registered prior to initiation of treatment no more than one working day prior to the planned start of treatment. **No exceptions will be permitted.**

Registration, ECOG Investigators: **A signed HHS 310 Form, a copy of the Institution's IRB-approved informed consent document, and written justification for any changes made to the informed consent for this protocol must be on file at the ECOG Coordinating Center before an ECOG Institutions may enter patients.** The signed HHS 310, institution informed consent, and investigators justification for changes will be submitted to the following address:

ECOG Coordinating Center
Frontier Science
Attn: IRB
303 Boylston Street
Brookline, MA 02146-7648

Patients must not start protocol treatment prior to registration.

15.6 M.D. Anderson Investigators

Specimen Submission. Blocks should be submitted within eighteen months after registration to the M.D. Anderson Cancer Center Cancer Tissue Bank directed by Dr. Aysegul A. Sahin. Blocks will be labeled with the M.D. Anderson number and the Southwest Oncology Group patient number and protocol number. The blocks should be accompanied by the Southwest Oncology Group Specimen Submission Form as well as a copy of the Surgical Pathology Report. The specimens should be submitted to:

Aysegul A. Sahin, M.D.
M.D. Anderson Cancer Center
Department of Pathology
Box 85
1515 Holcombe Boulevard
Houston, TX 77030
Phone: 713/794-1500

A copy of the Southwest Oncology Group Specimen Submission Form must also be submitted to the MDACC Research Office (Debbie Frye) for forwarding to the Southwest Oncology Group Statistical Center.

Storage of Blocks. Blocks will be numbered consecutively, and the identification number will be grouped by protocol and M.D. Anderson patient number. The submissions will be logged into the computerized data base and will undergo a prospective pathology quality control procedure prior to storage. The quality control procedure would entail a single H & E section to be certain that there is sufficient tumor cells present to confirm the diagnosis and to determine the histologic subtype, and to estimate the tumor grade by the Elston SBR and modified Black's nuclear grading criteria. The blocks will then be stored in a locked storage space in the Breast Tumor Tissue Bank. For medical/legal reasons, blocks will never leave the tissue repository except at the request of the original hospital. For later prognostic factor studies, slides will be cut by the central repository and distributed to other institutions if applicable.

Retrieval of blocks by originating institution. The bank will guarantee return of the blocks within 48 hours of a written request from the originating institution.

15.7 **Southwest Oncology Group Institutions** must enroll all **S9623** patients on the mandatory ancillary study, **S9719**, at the time of **S9623** registration, within the same registration phone call. If the patient refuses to give consent for **S9719**, the refusal must be documented on the initial **S9623** flow sheet. (See **S9719** protocol for information regarding submission of required samples.)

16.0 ETHICAL AND REGULATORY CONSIDERATIONS

The following must be observed to comply with Food and Drug Administration regulations for the conduct and monitoring of clinical investigations; they also represent sound research practice:

Informed Consent

The principles of informed consent are described by Federal Regulatory Guidelines (Federal Register Vol. 46, No. 17, January 27, 1981, part 50) and the Office for Protection from Research Risks Reports: Protection of Human Subjects (Code of Federal Regulations 45 CFR 46). They must be followed to comply with FDA regulations for the conduct and monitoring of clinical investigations.

Institutional Review

This study must be approved by an appropriate institutional review committee as defined by Federal Regulatory Guidelines (Ref. Federal Register Vol. 46, No. 17, January 27, 1981, part 56) and the Office for Protection from Research Risks Reports: Protection of Human Subjects (Code of Federal Regulations 45 CFR 46).

Drug Accountability

For each drug supplied for a study, an accountability ledger containing current and accurate inventory records covering receipt, dispensing, and the return of study drug supplies must be

treatment on this study will give you at least as good a chance as you might expect from other treatments.

We also want to find out what kind of side effects each of these treatment programs causes and how often they occur.

If you are being registered at a Southwest Oncology Group facility, you are also invited to take part in the ancillary study, S9719, "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy For Breast Cancer: Pilot Study to Evaluate Incidence." The purpose of this study is to learn whether the treatment for your breast cancer causes gene damage to your hematopoietic cells (early blood cells) which may be associated with development of leukemia in a small subgroup of breast cancer patients. No additional bone marrow examinations (others than those specified by this treatment protocol, S9623) will be required for participation in this companion study. If you participate, small amounts of your blood and bone marrow will be sent to a central laboratory for testing. *(9/1/98)* **Cells and genetic material will also be stored for possible future testing. The studies done on your blood cells may lead to discoveries which help future patients with breast cancer.** *(paragraph added ____)*

As part of the ongoing scientific and research activities of the Southwest Oncology Group and its agents or other participating cooperative groups, samples of your tissue may (with your approval and your physician's approval) be preserved and used for research and development purposes. If you also participate in S9719, samples of your blood (and possibly bone marrow) may be preserved for research and development purposes. By signing the S9719 portion of this consent form, you authorize the preservation and use of these blood (and marrow) samples taken from you. *(____)* **As a result of these research activities, an economic benefit may be derived directly or indirectly by the Southwest Oncology Group, individual researchers, and others engaged in these activities.**

We cannot and do not guarantee you will benefit if you take part in this study. The treatment you receive may even be harmful.

II. If you agree to take part in this study, the decision about your next treatment will be made by a process (called randomization) similar to picking numbers out of a hat.

If you are randomized to Arm 1, you will receive high-dose chemotherapy with three different drugs. *(3/15/97)* You will begin by receiving doxorubicin through a needle in your vein over about an hour. Your doctor may prefer to give the doxorubicin over a shorter time (about fifteen minutes). *(3/15/97)* Treatment with doxorubicin will be repeated every two weeks for a total of three cycles (treatment on Days 1, 15 and 29). Next, you will receive paclitaxel through a needle in your vein over a period of twenty-four hours. Treatment with paclitaxel will begin on Day 43 (fourteen days after the last dose of doxorubicin), and will be repeated every two weeks for a total of three cycles (treatment on Days 43, 57 and 71). Finally, you will receive cyclophosphamide through a needle in your vein over about an hour. Treatment with cyclophosphamide will begin on Day 85 (fourteen days after the last dose of paclitaxel), and will be repeated every two weeks for a total of three cycles (treatment on Days 85, 99 and 113). Beginning on Day 3 and continuing through Day 10 of each cycle of each of these drugs, you will also receive G-CSF as a daily shot just under your skin, and daily ciprofloxacin by mouth. These drugs are given to help prevent the side effects of chemotherapy.

If you are randomized to Arm 2, you will begin by receiving induction chemotherapy - doxorubicin and cyclophosphamide through a needle in your vein over about an hour. *(3/15/97)* Your doctor may prefer to give the doxorubicin over a shorter time (about fifteen minutes). *(3/15/97)* This treatment will be repeated every three weeks for a total of four cycles (Days 1, 22, 43 and 64). After the fourth cycle, you will have a period of time during which you will receive no treatment. During this time you will undergo harvest of stem cells from your bone marrow and/or your blood. *(11/1/96)* The manner of harvest may differ among hospitals. Within two weeks after your stem cells have been harvested, and within six weeks after your last dose of induction therapy, you will begin receiving high-dose chemotherapy to prepare you for stem cell infusion. The high-dose treatment that you will receive will be either STAMP I or STAMP V depending on the decision of the hospital where you are being treated.

In order to receive this treatment, you will require placement of a special central venous catheter. This will be a tube placed into a large vein in the chest. This tube can be of two basic types. (1) It can come out through the skin or (2) be attached to a small chamber with all of the device under the skin. Either one of these central vein catheters will allow the chemotherapy to be dripped in over a long period of time. In most patients, these can be placed under local anesthesia in an operating room and in most patients, these can remain in place indefinitely. Problems that can be associated with these catheters include pain, bleeding, infection, or clotting. In a minority of patients (< 10%), these problems lead to the catheter being removed.

Before you receive high-dose chemotherapy, cells will be collected to perform the transplant. These cells may be collected from the bone marrow, from the peripheral blood, or from both locations.

Bone marrow harvests are performed under general or spinal anesthesia. Multiple aspirations of liquid bone marrow are taken from the back of the pelvic bone. The amount of marrow removed depends on how rich it is in cells and how much you weigh. Collection of bone marrow cells usually takes about one hour and is associated with local discomfort which lasts several days and may require medication for pain. Bruising in this area is common. Infection in the skin or bone is exceedingly rare.

Collection of hematopoietic cells from the blood is performed by a technique called apheresis. You will receive one or more drugs called hematopoietic growth factors. These drugs will cause your white blood cell count to rise. Contained in these white blood cells are marrow cells which are capable of restoring your bone marrow function. After several days of hematopoietic growth factor administration, you will undergo one or more apheresis procedures. Blood will be withdrawn through a central catheter, which has already been inserted, and will flow into the apheresis machine. *(11/1/96)* This machine will spin the blood and will collect the white blood fraction. The remaining blood elements will be returned to you through your central venous catheter. *(3/15/97)* Apheresis is usually very well tolerated. It is possible, however, to experience some lightheadedness at the beginning of the procedure. Occasionally, patients may feel tingling or numbness of the hands or feet. *(11/1/96)* Infection of the central catheter during apheresis is very unusual. *(11/1/96)*

STAMP I

You will receive cyclophosphamide and cisplatin through your catheter for three days in a row. Cyclophosphamide will be given over an hour each day. Cisplatin will be given continuously (24 hours a day). On the fourth day, you will receive BNCU (carmustine) through your catheter over two hours. On the seventh day, your stem cells will be infused. (The manner of infusion and supportive care after the infusion may differ among hospitals.)

STAMP V

You will receive cyclophosphamide, thiotepa and carboplatin through your catheter continuously (24 hours a day) for four days. On the eighth day, your stem cells will be infused. (The manner of infusion and supportive care after the infusion may differ among hospitals.)

Regardless of which treatment you receive, you will also have radiation therapy if you have had a lumpectomy before entering this study. (Your doctor may also advise radiation therapy in certain cases if you received a mastectomy.) Radiation therapy to the chest area will be given four to six weeks after your last cycle of chemotherapy, and will most likely be given once a day, five days per week, for six weeks.

Regardless of which treatment you receive, if you have gone through menopause (either naturally or because of surgery) or if your tumor contained hormone receptors and you have not gone through menopause you will begin taking tamoxifen four weeks after the completion of chemotherapy and will continue taking tamoxifen once a day by mouth for five years.

You will have x-rays, laboratory tests and other tests, including blood tests, during your therapy so your doctor can see how your body is responding to treatment.

For patients participating on **S9719**: As part of your treatment on **S9623**, you will have cells collected from the bone marrow, from the peripheral blood, or from both locations. Part of the bone marrow and blood samples taken for your treatment on this study may be submitted for **S9719**. You will also have peripheral blood taken at the time of apheresis collection and at 3 and 12 months following completion of all chemotherapy. *(9/1/98)* This material will be sent to a centralized laboratory where it will be tested for genetic damage. Also, if a secondary cancer is diagnosed any time after you begin treatment, you are requested to have bone marrow and blood submitted at that time. *(9/1/98)* The submission of these samples is very important for this study and will help in finding out if genetic damage is related to a second cancer. *(paragraph added _____)*

There are circumstances under which your doctor might be required to discontinue or delay your treatment whether you agree or not. These circumstances include: your tumor gets worse despite the treatment; the side effects of this treatment are too dangerous for you; new information becomes available which suggests that this treatment will be ineffective or unsafe for you.

BCNU, carboplatin, ciprofloxacin, cisplatin, cyclophosphamide, doxorubicin, G-CSF, paclitaxel, tamoxifen and thiotepa are commercially available.

Patients with a pre-existing history of such problems should discuss the indication for tamoxifen treatment carefully with their physician.

Tamoxifen may be harmful to be unborn fetus, and pregnancy should be avoided while taking this medication and for at least two months after its discontinuation.

Thiotepa

This drug can affect several organs (or parts) of your body in addition to the cancer cells. Stomach upset is common which can cause you to experience loss of appetite, nausea and/or vomiting; this is seldom severe. Temporary hair loss (not only from the scalp but possibly underarms, beard, eyelashes and pubic area) can occur. Pain at the site of injection is also common.

Other effects may include dizziness, headache, interruption of the menstrual cycle, interference in the production of sperm, and fever. Allergic reactions are rare, but may include hives and skin rash. Difficulty breathing and inflammation of the urinary bladder are also possible side-effects.

Thiotepa can decrease the blood cells produced in the bone marrow. This can lead to:

1) decreased white cells which may make you more vulnerable to infection.

2) lower number of red cells which can give you symptoms of shortness of breath, weakness and fatigue.

3) lower platelets which can result in easy bruising or bleeding for a longer time.

The drug's effect on the bone marrow is usually only temporary and transfusions are available if needed to counteract decreases in these cells until your bone marrow recovers. Blood samples will be taken frequently to monitor these effects of the drug on your bone marrow. Death from severe infection and bleeding as a result of decreased blood counts has occurred.

The occurrence of acute leukemia has been reported rarely in patients treated with combination chemotherapy using this type of drug.

Breast, Upper Chest Wall, and Regional Lymph Nodes

Side effects which have been observed in some people undergoing this type of radiotherapy include skin reaction in terms of dryness, redness and peeling of the skin; and soreness on swallowing - these effects are temporary. There is also a possibility of inflammation of the lungs and a drop in white blood count, platelet count and red blood cells. There is also a possibility of cardiac problems secondary to the radiation; however, the chances of this occurring are slim.

IV. There may be other treatments for your cancer, such as other (conventional) chemotherapy treatments, radiotherapy, hormonal therapy, surgery or bone marrow transplantation. Your doctors feel that your treatment on this study will give you at least as good a chance as you might expect from other therapies.

For patients participating in **S9719**: There may be other ways of determining if there is genetic damage in your cells. The methods used in **S9719** are comparable to others that may be available. You also have the option of not having this procedure done on your blood and bone marrow samples. *(9/1/98) (paragraph added____)*

V. If you are pregnant, you cannot take part in this study. You will take a urine test to see if you are pregnant before you start treatment. If you are sexually active, we strongly recommend you take precautions to avoid the possibility of becoming pregnant because we do not know how these drugs could affect an unborn child.

VI. If you experience illness as a result of treatment on this study, you will/will not (circle one) receive free emergency medical treatment. We cannot give you free continuing medical care and/or hospitalization, nor can we pay you to take part in this study.

VII. We will keep any information we learn from this study confidential and disclose it only with your permission. By signing this form, however, you allow us to make your records available to the National Cancer Institute, the Food and Drug Administration, and the Southwest Oncology Group (as well as other participating cooperative groups). If we publish the information we learn from this study in a medical journal, you will not be identified by name.

For patients participating on **S9719**: If you agree to participate on **S9719**, you allow us to make your records available to the National Cancer Institute, the Food and Drug Administration, the U.S. Army Medical Research and Material Command and the Southwest Oncology Group. If we publish the information we learn from this study in a medical journal, you will not be identified by name. You may request a copy of the study results after the study is finished. *(paragraph added _____)*

VIII. Whether or not you take part in this study will not affect your future relations with your doctors (there will be no loss of benefit or change in attitude) or _____ (hospital name). If significant new findings are developed during the course of this study which may relate to your willingness to continue, this information will be provided to you. In addition, you understand that you may refuse to continue on this study, at any time after the start of therapy, without fear of prejudice to additional treatment that may be needed.

IX. The doctor(s) involved with your care can answer any questions you may have about the drug program. In case of a problem or emergency, you can call the doctors listed below day or night.

	Office	Home
Dr.		
Dr.		
Dr.		

You can also call the Institutional Review Board (#_____) if you have any questions, comments or concerns about the study or your rights as a research subject.

X. We will give you a copy of this form to keep.

XI. You are deciding whether or not to take part in this study. If you sign, it means that you have decided to volunteer after reading and understanding all the information on this form.

_____	_____
Date	Signature of Subject
_____	_____
Signature of Witness	Signature of Investigator
_____	
Time	

XII. **Consent for ancillary study S9719**, " CLONAL HEMATOPOIESIS AS A MARKER OF GENETIC DAMAGE FOLLOWING ADJUVANT CHEMOTHERAPY FOR BREAST CANCER: PILOT STUDY TO EVALUATE INCIDENCE".

You are deciding whether or not to take part in this study. If you sign, it means that you have decided to volunteer after reading and understanding all the information on this form.

Date

Signature of Subject

Signature of Witness

Signature of Investigator

Time

EXHIBIT 2

Date: 8/10/99
Sender: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>
To: Marilyn Slovak, 'TAMRA ONER' <toner@swog.org>
Priority: Normal
Receipt requested
Subject: DAMD17-97-1-7088

I just wanted to let you know your draft amendment is still being worked on but will be approximately a month until we are able to obtain a decision for you. . According to COL Zadinsky, Deputy Chief of Staff for Regulatory Compliance and Quality, the amendment must go before the Board for approval. The next board does not meet until September 8. During this period, Louise Pascal may be contacting you for additional information/documentation. As soon as I obtain a decision, I will contact you immediately.

Karen

Karen Stotler
Contract Specialist
U.S. Army Medical Research Acquisition Activity
ATTN: MCMR-AAA-B
820 Chandler Street
Fort Detrick, MD 21702-5014
Telephone: 301/619-6857
Fax: 301/619-4084

Date: 7/14/99
Sender: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>
To: Marilyn Slovak
Priority: Normal
Receipt requested
Subject: DAMD17-97-1-7088, SWOG Study

Dr. Slovak,

I received your voice message. I apologize for not responding sooner. I have contacted our Human Subjects Protection Division on numerous occasions and cannot obtain an answer for you and Tamra Oner. This morning I sent COL Zadinsky, the Department Head, an e-mail stating I cannot get anyone in her department to return my calls or answer my e-mails and urgently requesting we get you an answer today.

Please be a little patient with me while I continue to work on this internal problem. Hopefully I will have an answer by the end of the day for you.

Thank you again,

Karen

Karen Stotler
Contract Specialist
U.S. Army Medical Research Acquisition Activity
ATTN: MCMR-AAA-B
820 Chandler Street
Fort Detrick, MD 21702-5014
Telephone: 301/619-6857
Fax: 301/619-4084

Reply Separator

Subject: RE: Receipt of 7/14/99 11:03 AM message
Author: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>
Date: 7/15/99 6:14 AM

Dr. Slovak,

COL Zadinsky is out of the office until Monday but responded to my call for assistance. The COL sent my message to LTC Pierson, who is covering for the COL in her absence. Hopefully I will have an answer for you today.

Karen

-----Original Message-----
From: mslovak@smtplink.Coh.ORG [mailto:mslovak@smtplink.Coh.ORG]
Sent: Wednesday, July 14, 1999 7:45 PM
To: Karen.Stotler@DET.AMEDD.ARMY.MIL
Subject: Receipt of 7/14/99 11:03 AM message

Re:DAMD17-97-1-7088, SWOG Study

Date: 7/30/99
Sender: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>
To: Marilyn Slovak
Priority: Normal
Receipt requested
Subject: RE: Follow-up from June 15th

Dr. Slovak,

I forwarded your e-mail to COL Zadinsky and once again requested an answer for you. I don't understand why you have not been contacted by our Human Subjects Protection Division or why I haven't received a reply to my request. I will keep on this, but until I receive an answer, I cannot do anything.

Karen

-----Original Message-----
From: mslovak@smtplink.Coh.ORG [mailto:mslovak@smtplink.Coh.ORG]
Sent: Friday, July 30, 1999 11:59 AM
To: Karen.Stotler@DET.AMEDD.ARMY.MIL; toner@swog.org
Subject: Re:Follow-up from June 15th

Dear Ms. Stotler,

I have not received a response from the DOD regarding the amendment proposal to DAMD17-97-1-7088. When I contacted you two weeks ago, you were under the impression that a Col Zadinsky would respond the following workday, however, I have not heard from either of you to date.

Our proposed protocol amendment is necessary to increase patient accrual to the clonal hematopoiesis project. We have put a temporary hold on the SWOG study and DOD funds until we obtain DOD and CTEP approval for this amendment. After approval, we will have another minor delay to get the amendment through the individual SWOG institutional IRBs. I am also not sure how to respond to the required June meeting/abstract, and progress report. I would like to obtain a letter from the DOD indicating that we are working together to increase patient accrual and bring this project to completion despite the "internal" problems. I hope to hear from you soon. Please advise.

Marilyn L. Slovak, Ph.D., FACMG
City of Hope National Medical Center
Department of Cytogenetics, Northwest Building Room 2255
1500 East Duarte Road
Duarte, CA 91010
phone: 626/359-8111 ext. 2313
fax: 626/301-8877
mslovak@coh.org

Reply Separator

Date: 8/2/99
Sender: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>
To: Marilyn Slovak
Priority: Normal
Receipt requested
Subject: RE: Follow-up from June 15th

Dr. Slovak,

In response to my e-mails to COL Zadinsky and LTC Jerry Pierson, your amendment is currently being worked on. I am supposed to receive an update on the timeline of what has been done and what is needed to be done today. According to COL Zadinsky, this amendment is not something they can quickly sign off on. Hopefully I will be able to give you some information later today on when you can expect to receive an answer from us.

Thank you for being patient.

Karen Stotler

-----Original Message-----
From: mslovak@smtplink.Coh.ORG [mailto:mslovak@smtplink.Coh.ORG]
Sent: Friday, July 30, 1999 11:59 AM
To: Karen.Stotler@DET.AMEDD.ARMY.MIL; toner@swog.org
Subject: Re:Follow-up from June 15th

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Marilyn L. Slovak, Ph.D., FACMG
City of Hope National Medical Center
Department of Cytogenetics, Northwest Building Room 2255
1500 East Duarte Road
Duarte, CA 91010
phone: 626/359-8111 ext. 2313
fax: 626/301-8877
mslovak@coh.org

EXHIBIT 3

Date: 9/9/99
Sender: TAMRA ONER <toner@swog.org>
To: Pascal Louise M <Louise.Pascal@DET.AMEDD.ARMY.MIL>
cc: Stotler Karen S <Karen.Stotler@DET.AMEDD.ARMY.MIL>, Peter Ravdin <pravdin@swog.org>, Marilyn Slovak, DANA SPARKS <dsparks@swog.org>
Priority: Normal
Subject: RE: Follow up Dr. Coltman's protocol

OFFICE MEMO Subject: RE: Follow up Dr. Coltman's protocol Time: 10:29 AM Date:9/9/1999

Hi Louise - thank you for the update. We'll await further information regarding the items to be addressed.
Best regards, Tamra

Pascal Louise M wrote:
>Dear Tamra,
>
>Just a brief note to first thank you and Dr. Ravdin for your assistance and
>availability regarding the HSRRB meeting of 8 Sept. Your coordination and
>support has been very helpful in moving this process forward. Also, Dr.
>Ravdin participation yesterday proved very beneficial.
>
>Secondly, as an update, Dr. Coltman's protocol was given conditional
>approval. Please be aware this is unofficial- the HSRRB minutes are
>prepared, reviewed and approved. Thereafter, Dr. Coltman and you will be
>receiving correspondence with the formal notification of the "conditional
>approval" of the protocol. There are a few items that will need to be
>addressed before the final letter of approval can be issued from this
>office. These few items can be addressed directly with me or the Acting
>Chair of the HSRRB (COL Zadinsky) - these items do not require a return to a
>convened meeting of the HSRRB. After all items are resolved, the final
>letter will be issued - upon receipt of the final approval letter, the
>enrollment of additional subjects can begin.
>
>Again, I appreciate your help. Please contact me if you have any questions.
>
>Thank you, Louise

Tamra N. Oner
Protocol Coordinator
Southwest Oncology Group
14980 Omicron Drive
San Antonio, TX 78245
Phone: (210) 677-8808
Fax: (210) 667-0006
Email: toner@swog.org

S9719 Biologic

Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence

Study Coordinators:
M Slovak, C Willman, W Stock, K Albain, S Bearman

Date Activated:
10/15/97

Statisticians:
S Green, D Lew

Data Coordinator:
K Rantala

Objectives

To estimate the incidence of early genetic damage, defined by the presence of clonal hematopoiesis using a general clonality assay, the HUMARA (human androgen receptor assay), in pretreatment blood and bone marrow, apheresis, and two sequential post-treatment specimens in breast cancer patients enrolled in S9623.

To detect genetic damage following dose-intensive adjuvant regimens for breast cancer by screening these samples for the presence of defective DNA mismatch repair mechanisms and loss of heterozygosity using microsatellite instability assays.

To estimate the incidence of MLL (myeloid lymphoid leukemia) gene fusion transcripts in cases where either the HUMARA or microsatellite repeat assays are positive for clonal hematopoiesis, a finding commonly reported in t-AML with 11q23 abnormalities.

To determine the frequency of RAS gene mutations (H-, K-, and N-RAS) following dose-intensive adjuvant regimens for breast cancer.

Patient Population

Patients must be enrolled on S9623 and registered to this biologic study before beginning protocol treatment on S9623.

A pretreatment sample of peripheral blood, and bone marrow when available, must be collected.

Accrual Goals

One hundred patients per arm from S9623 will be accrued on this study.

Summary Statement

As of June 30, 1999, 13 patients had been registered to this study.

A September 1, 1998, amendment eliminated the requirement for buccal mucosa samples, made the pre-treatment bone marrow sample optional, and reduced the amount of blood collected. Funds are now available to offset mailing costs for the blood and bone marrow samples.

Enrollment to this study will become mandatory for patients registering to S9623 from SWOG institutions.

Registration by Institution

Registrations ending June 30, 1999

Institutions	Total Reg
Columbia River CCOP	3
Oregon Hlth Sci Univ	2
St Francis/Stormont/Kansas, U of	2
Arizona, U of	1
Harris Methodist/San Antonio, U of TX	1
Henry Ford Hosp	1
N Colorado Med Ctr/Colorado, U of	1
Northwest CCOP	1
Salem Hospital/Oregon Hlth Sci Univ	1
Total (9 Institutions)	13

S9623 Phase III Intergroup

Coordinating Group: SWOG

A Comparison of Intensive Sequential Chemotherapy using Doxorubicin plus Paclitaxel plus Cyclophosphamide with High Dose Chemotherapy and Autologous Hematopoietic Progenitor Cell Support for Primary Breast Cancer in Women with 4 - 9 Involved Axillary Lymph Nodes

---

Intergroup Participants:
SWOG, CALGB, ECOG, MDACC, NCCTG

Date Activated:
7/1/96

Study Coordinators:
S Bearman, L Pierce, W Peters, J Ingle (NCCTG),
K Holland (ECOG), C Hudis (CALGB),
Z Rahman (MDACC)

Statisticians:
S Green, D Lew

Data Coordinator:
K Rantala

Schema

Title:
h:\groups\swogadm\schemas\sch9623.eps
Creator:
ImageMark Software Labs
Preview:
This EPS picture was not saved
with a preview included in it.
Comment:
This EPS picture will print to a
PostScript printer, but not to
other types of printers.

Objectives

To compare induction chemotherapy with doxorubicin and cyclophosphamide, followed by high dose chemotherapy and autologous hematopoietic progenitor cell support vs intensive sequential chemotherapy using doxorubicin, paclitaxel and cyclophosphamide with G-CSF support with respect to disease-free survival and overall survival in operable breast cancer patients with 4-9 positive nodes.

To compare toxicity for patients treated with high-dose chemotherapy with toxicity for patients treated with intensive sequential chemotherapy.

Patient Population

This study is open to patients with histologically confirmed adenocarcinoma of the female breast with 4-9 histologically confirmed involved axillary lymph nodes. Patients must not have N3, T4, or M1 disease. Patients must have undergone a breast-sparing procedure or mastectomy with at least 10 nodes sampled within 12 weeks prior to registration. Surgical margins must be negative for invasive and non-invasive ductal carcinoma.

Patients must have received no prior chemotherapy for any malignancy, and no prior radiotherapy to the breast.

Patients must have adequate hematologic, renal, hepatic, pulmonary, and cardiac function, and must be HIV negative. Patients must have a SWOG performance status of 0-1 and must not have any serious medical or psychiatric illness. All patients must have been evaluated and approved for this study, prior to registration, by a transplant center approved for this study by one of the participating cooperative groups.

Stratification/Descriptive Factors

Patients will be stratified by primary treatment: mastectomy with no RT vs mastectomy + RT following chemotherapy vs breast sparing procedure + RT following chemotherapy. Patients will be described by (1) menopausal status: pre vs post vs other; (2) estrogen receptor status: ER positive vs ER negative vs unknown; (3) progesterone receptor status: PgR positive vs PgR negative vs unknown; (4) N2 disease: yes vs no; (5) T3 disease: yes vs no; (6) transplant regimen: STAMP I vs STAMP V; and (7) source of progenitor cells: marrow vs peripheral blood vs both.

Accrual Goals

The accrual goal is 1000 eligible patients. Two interim analyses will be performed, after approximately one-third and two-thirds of the expected number of events have occurred.

Summary Statement

This study was activated on July 1, 1996. As of June 30, 1999, 504 patients had been enrolled. One hundred twelve patients are currently ineligible, 92 due to missing baseline data. Seventy-five of these could become eligible; institutions are reminded to submit overdue data. The most common other reasons for ineligibility were positive margins (6 patients), insufficient number of nodes sampled (4), and transplant center not approved by SWOG (3).

Seventy-one out of 112 patients evaluated for toxicity on the sequential chemotherapy arm (including five patients currently ineligible due to overdue data) experienced Grade 4 hematologic toxicities. Thirteen experienced Grade 4 non-hematologic toxicity, the most common being myalgia/arthralgia in four.

Three patients on the high dose chemotherapy arm have died due to toxicity. One patient died during induction due to ARDS and infection. Two patients died due to transplant toxicities: one due to complications from VOD, and the other due to pneumonia ("Respiratory infection w/o neutropenia"). One hundred fourteen patients were evaluated for toxicity due to induction on the high dose chemotherapy arm (including five patients currently ineligible due to overdue data); 78 experienced Grade 4 toxicities as maximum degree. Eleven of these experienced non-hematologic Grade 4 toxicities. The Grade 4 "Cardiovascular - other" toxicity was cardiac arrest and the Grade 4 "GU - other" was renal tubular damage.

Seventy-nine of the 87 patients evaluated for toxicity post-transplant experienced Grade 4 toxicities as maximum degree, five with non-hematologic Grade 4. The Grade 4 "Liver – other" toxicity was cholecystitis and the Grade 4 "GU – other" was hemolytic uremic syndrome.

A new amendment to this study will require that consent for enrollment on the ancillary study S9719 be requested for patients from SWOG institutions. Another amendment incorporates S9702 into S9623.

City of Hope National Medical Center
Anatomic Pathology Policy and Procedure Manual

Subject: HUMARA Assay		
Dept: Cytogenetics	**Section: Staining**	**Reviewed Date:**
Pages: 16	**File name: CYGN-315.doc**	
Effective Date: 08-28-97 **Revised Date: 03-18-99**	**Approved by:** Marilyn L. Slovak 3/18/99	

Principle:

The occurrence of clonal hematopoiesis can be determined by screening for X-linked polymorphism of the human androgen receptor gene.

Specimen Size and Storage

Four 7 ml sodium heparin tubes (green top) should be collected from each patient for HUMARA analysis. T-cell separation can also be performed on BM sample, but a 200 µl aliquot should be isolated using the Qiagen kit in addition to the T-cell and PMN isolation. For example, if you receive 2 ml of bone marrow. Remove 200 µl for Qiagen isolation and run the remaining sample over the Ficolll column for T-cell and PMN isolation.

Ideally, blood should be processed the sam e day as collection, however if storage is necessary blood and bone marrow should be stored at 4°.

Procedure:

A. Collection of Somatic Tissue from Buccal Mucosa Scraping

1. Rinse mouth thoroughly with water.
2. Firmly brush inside of cheek with a *CytoSoft* Cytology Brush no. CYB-1 using approximately ten strokes.
3. Dislodge cells from brush into 10 ml of 1x PBS contained in a 15 ml polystyrene tube.
4. Repeat steps 2-3 3x alternating between sides of cheeks, using a new brush each time and the same collection tube.

B. DNA Isolation

1. Buccal Somatic Tissue:

a. Pellet cells by centrifuging for 7 minutes at 1400 rpm in a tabletop centrifuge.
b. Remove all but 200 µl of supernatant.
c. Resuspend cells and aliquot into a microcentrifuge tube.

Leukocyte Fraction of Peripheral Blood or Bone Marrow:
a. Centrifuge 7 ml of peripheral blood for 7 minutes at 1400 rpm.
b. Carefully remove intermediate leukocyte enriched layer with a Pasteur pipette and place into a 15 ml polystyrene tube.
c. Aliquot 200 µl of leukocyte fraction into a microcentrifuge tube.

2. To a 200 µl cell aliquot, add 20 µl RNase A (20 mg/ml) and incubate for 2 minutes at room temperature.

3. Add 25 µl proteinase K (20 mg/ml) and 200 µl buffer AL, and immediately vortex for 15 seconds. Incubate 10 minutes at 70° C if starting material was buccal tissue or whole blood, 15 minutes if starting material was an apheresis sample, and 20 minutes if starting material was bone marrow or a leukocyte fraction.

4. Add 210 µl of 100% ethanol, and mix again by vortexing.

5. Place a QIAamp spin column into a 2 ml collection tube [spin columns and collection tubes are provided in the kit from QIAGEN (no. 29104)]. Carefully apply mixture from step 4 to the spin column without moistening the rim, and centrifuge for 1 minute at 8000 rpm in microcentrifuge.

6. Discard filtrate and place spin column in a clean collection tube. To wash spin column membrane, add 500 µl buffer AW to spin column and centrifuge for 1 minute at 8000 rpm. Repeat wash 1x, with a second final spin for 2 minutes at 14,000 rpm.

7. Discard filtrate and place spin column in a clean collection tube. To elute DNA, add 50 µl of distilled water (preheated to 70° C) to the spin column and incubate loaded spin column for 5 minutes at 70° C.

8. Centrifuge for 2 minutes at 8000 rpm, followed by a final spin for 30 seconds at 14,000 rpm.

9. Place filtrate into a 1.5 ml microcentrifuge tube and determine DNA concentration.

T-Cell and PMN Isolation

1. Label two 50 ml conical tubes.

2. Pour blood from 2 sodium heparin tubes into one of the 50 ml tubes with total sample volume of 14 to 15 ml. Repeat for the second set of sodium heparin tubes.

3. Add 14 ml RPMI to each 50 ml conical. Volume should equal ~30 ml. Pipette to mix.

4. Pipette 15 ml of Histo-Paque 1077 into the second 50ml tube.

Subject: HUMARA Assay

5. Using a 25 ml pipette, slowly lay blood/RPMI on top of Histo-Paque layer.

6. Spin at 1200 rpm for 40 minutes at room temperature.

7. Aspirate monolayer and transfer to a 15ml polystyrene tube. To assure you have the complete mononuclear layer, aspirate ~14 ml of serum/monolayer/Histopaque to the 15 ml tube.

8. Spin at 1200 rpm for 10 minutes.

9. Decant supernatant and resuspend pellets in 7 ml RPMI combine the mononuclear pellets in one 15 ml polystyrene tube. If sample needs to be stored overnight, resuspend pellet in 7ml RPMI/5% FBS and store at 4°. Proceed with protocol as usual the next morning.

10. Spin at 1200 rpm for 10 minutes. During this spin, wash Dynabeads in the cold room.
    a. Place 7 µl of Dynabeads into a 0.5 ml microcentrifuge tube.
    b. Add 250 µl 2%FBS diluted in PBS, place tube on the magnet and remove supernatent using a 200 µl pipette.
    c. Repeat wash.

11. Decant supernatent. From this point all T-cell isolation steps should be completed in the cold room.

12. Resuspend pellet in 250 µl PBS/ 2% FBS and transfer sample to tube containing washed Dynabeads.

13. Place on rotor in the cold room for 30 minutes. At this point, continue with PMN isolation.

14. Aspirate remaining Histo-Paque in 50 ml tube with a pipette.

15. Resuspend pellet and add Triton X working solution up to a final volume of 45 ml.

16. Invert tube 2-3 times.

17. Spin at 3000 rpm for 10 minutes.

18. Decant supernantant and resuspend pellet in 25 ml Triton X working solution.

19. Spin at 3000 rpm for 10 minutes and decant supernatant.

20. Add 2.25 ml 75mM NaCl/25mM EDTA buffer and resuspend pellet.

21. Transfer to a 15 ml polypropylene tube. Add 250 µl Proteinase K/ SDS. [2 mg/ml in 5% SDS]

22. Incubate tube at 37°C overnight or 55° for 2 hrs.

23. At this point finish T-cell isolation.

24. Remove T-cell tube from rotor and place tube on magnet.

25. Wait 30 seconds and remove supernatant with a 200 µl pipette.
26. Add 300 µl PBS/ 2% FBS and mix well.
27. Repeat 4 times.
28. Remove final supernatant and add 300 µl ddH_20 to dynabeads.
29. Transfer to a 1.5 ml tube, add 60 µl Proteinase K/ SDS and digest overnight in 37° water bath or 2 hrs at 55°.

DNA Extraction from PMN

1. Remove samples from water bath.
2. Add 2 ml of 1:1 phenol/chloroform mixture.
3. Mix well and place on rotor for 30 min. to 1 hour.
4. Spin at 3000 rpm for 10 minutes. Remove aqueous layer to fresh polypropylene tube.
5. Repeat phenol/chloroform extraction.
6. Add 2.5 volumes of -20°C 100% EtOH. (~5 ml)
7. Add 1/3 volume of 10M NH_4OAc. (~660 µl)
8. Mix and DNA strands should appear.
9. Place tube in –80°C freezer overnight. One hour is sufficient but overnight precipitation will increase yield.
10. Transfer DNA to labeled eppendorf tube.
11. Spin 14,000 rpm at 4°C for 30 minutes.
12. Wash with 1 ml 70% EtOH.
13. Spin at 14,000 rpm at 4°C for 30 minutes.
14. Carefully pour off EtOH.
15. Air dry for one hour.
16. Resuspend in 200 µl PCR H_2O.
17. Place in 37°C water bath overnight to elute DNA.

Subject: HUMARA Assay

18. Determine DNA concentration and add water if necessary to obtain ideal concentration of 100 ηg/μl. 10 μl are used in the Rsa I and Hpa II digestion which, at ideal concentration, equals a total of 1 μg DNA per digest.

DNA extraction from T-cells

1. Remove samples from water bath.
2. Add 140 μl ddH_2O to sample.
3. Add 500 μl 1:1 phenol/chloroform. Label this tube P/C 1.
4. Mix well and place on rotor for 30 min. to 1 hour.
5. Spin at 3000 rpm for 10 minutes.
6. Transfer supernatant, up to interface, to a new eppendorf tube (P/C 2). Do not take the white dirty layer.
7. Perform a back extraction by adding 250 μl TE buffer to P/C 1 and placing the tube back on the rotor for 1 hour. Set aside the aqueous supernatent (P/C 2) separated in step 6.
8. Spin the back extraction at 3000 rpm for 10 minutes. Transfer supernatent up to interface to the same eppendorf tube in step 6.
9. Add 600 μl phenol: chloroform to P/C 2 and place back on rotor for 30 min. to 1 hour.
10. Spin at 3000 rpm for 10 minutes and transfer supernatent to a new eppendorf tube.
11. Separate supernatent into 2 eppendorf tubes (350 μl each)
12. Precipitate DNA by adding 2 volumes-20° C 100% EtOH (700 μl), 1/3 volume 10 M NH_4OAc (130 μl), and 1 μl glycogen.
13. Gently mix and place in -80°C freezer overnight.
14. Spin at 14,000 rpm at 4°C for 30 minutes.
15. Carefully pour off supernatant.
16. Wash with 1 ml 70% EtOH
17. Spin at 14,000 rpm at 4°C for 30 minutes.
18. Pour off 70% EtOH.
19. Air dry for 1 hour.

20. Resuspend in 15 µl PCR H_2O.

21. Place in 37°C water bath overnight.

22. Determine DNA concentration.

C. Determination of DNA Concentration

1. Prepare a 100-fold dilution for each DNA sample (1 µl DNA + 99 µl ddH_2O) and a "blank" sample (100 µl ddH_2O).

2. To use the Beckman DU-64 Spectrophotometer, follow steps outlined below (also refer to manufacturer's instructions):

   a. Press UV key and wait until display reads "UV" instead of "uv" (approximately 5 minutes).

   b. Press PROG key and select "program 7:260/280" option (selection is made by scrolling using PROG key).

   c. Press R/S key and then the ENTR key. The display will read "insert blank: R/S".

   d. Rinse micro cell cuvette (height 8mm; Beckman Instruments, Inc.) 3x with distilled water, pipette "blank" prepared in step1 into cuvette, and wipe the glass sides with a kimwipe to remove fingerprints.

   e. Insert cuvette into cuvette holder of spectrophotometer (be sure to orient cuvette properly in accordance with the light beam) and press R/S key to calibrate machine.

   f. After the display reads "insert sample: R/S", remove cuvette and rinse 3x with distilled water.

   g. Pipette diluted DNA sample into cuvette, wipe glass sides with kimwipe, and repeat step e. After pressing R/S key, the amount of light absorbed by the sample at 260nm and 280nm will be tabulated and displayed.

   h. Repeat steps f and g for remaining samples.

   i. Calculate DNA concentration and purity using the following formulas:

      DNA concentration (ug/ul) = A_{260} x 0.05 µg/µl x dilution factor

      DNA purity = A_{260}/A_{280}

Note: DNA concentration can be determined spectrophotometrically because nucleotide nitrogenous bases absorb light maximally at 260nm (A_{260}). An A_{260} value of 1 corresponds to 50

μg/ml of double-stranded DNA. Since protein has a maximum absorbance at 280nm (A_{280}), the purity of DNA can be assessed by determining the A_{260}/A_{280} ratio. The A_{260}/A_{280} ratio for pure DNA is 1.95, although 1.75-2.0 is acceptable. A very low or high A_{260}/A_{280} ratio is indicative of protein or RNA contamination, respectively.

D. Digestion of Genomic DNA

1. For each DNA prepare 2 digestion tubes; one with Rsa I alone and one with Rsa I/ Hpa II.

Component	Rsa I	Rsa I and Hpa II
Rsa I	0.5 μl (20 units)	0.5 μl (20 units)
Hpa II	--------	0.77 μl (44 units)
Human DNA	10 μl (10ηg- 1μg)	10 μl (10ηg- 1μg)
10X L buffer	2 μl	2 μl
ddH_20	7.5 μl	6.73 μl

2. Incubate digestion mixture in a 37°C water bath overnight.

3. Store digested DNA at 4°C.

E. Polymerase Chain Reaction (PCR)

1. Aliquot 2 μl digested DNA to respective PCR tubes.

2. Prepare Master Mix in a foil covered tube:

Component	Volume per reaction
PCR H_2O	10.75 μl
10x SIGMA PCR buffer II	2.50 μl
2.5 mM dNTP	2.00 μl
*Humara I (5 pmol/μl)	2.50 μl
Humara II (5 pmol/μl)	2.50 μl
100% DMSO	1.25 μl
1.5 mM $MgCl_2$	1.50 μl
SIGMA Taq (5 units/ μl)	0.10 μl
Total Volume of Master Mix	23.10 μl

* Fluorescein Tagged Primer

3. Place reaction in thermal cycler. Use "hum/ggil" program under user option "Vicki".
[5 min. 94°C, 29 cycles (45 seconds 94°C, 30 seconds 60°C, 30 seconds 72°C), 10 minutes 72°C, hold at 4°C.]

4. Store reactions at -20°C and keep fluorescent products away from light.

5. If initial PCR does not work a nested PCR should be prepared. This PCR is not labeled and is not light sensitive.

   a.

Component	Volume per reaction
PCR H_2O	11.75 µl
DNA	1.00 µl
Humara III (5 pmol/µl)	2.50 µl
Humara IV (5 pmol/µl)	2.50 µl
10x Buffer	2.50 µl
2.5 mM dNTP	2.00 µl
100% DMSO	1.25 µl
1.5 mM $MgCl_2$	1.50 µl
SIGMA Taq (5 U/µl)	0.10 µl

   Nested PCR Thermocycler Program: "humnest" under user option "Vicki"
   94°C – 5 minutes, 20 cycles (94°C – 45 seconds, 60°C – 30 seconds, 72°C – 30 seconds) 72°C - 10 minutes, 4°C – hold.

   b. Dilute amplified PCR products 1:25. [2 µl PCR product + 48 µl PCR H_2O.]
   c. Use 2 µl diluted product as template DNA in fluorescent PCR.

F. Sequencing Gel

1. Remove any dust from inner surfaces of glass plates for sequencing (21 x 40cm; Bio-Rad) by wiping with kimwipes.

2. Inside hood, thoroughly clean inner surfaces of glass plates by siliconizing with Sigmacote (Sigma). Pour approximately 2 ml of Sigmacote onto each plate and through firm swipes, evenly distribute over entire area of plate.

3. Run distilled water over inner surfaces with a squirt bottle to confirm cleanliness. Water should run off the plate and not "stick" if plates are clean.

4. Allow plates to air dry for approximately 5 minutes in an upright position by propping plates against a vertical object (e.g. wall or window) with inner surface of plates facing the object. Remove any residual water drops from innner surfaces by blow drying with an air duster (Dust Off).

5. Assemble glass plates according to manufacturer's instructions. Use a 34 toothed 0.75 mm comb (BioRad). Clamp comb to plates.

6. Prepare a 4% or 6% acrylamide-urea denaturing gel by mixing 52ml Sequagel-4 (6) gel solution, 13ml Sequagel-Complete buffer reagent, and 520ul freshly prepared 10% ammonium persulfate in a

100ml beaker. Add ammonium persulfate last and immediately before injection between the glass plates since this chemical compound initiates polymerization.

7. Without creating bubbles, slowly and carefully inject gel material between glass plates to the top using a 60cc syringe. Make certain the gel material penetrates spaces between the teeth of comb. Gel will fully polymerize in 2 hours.

8. Gel can be stored overnight at 4°C (cold room). Prop gel apparatus at an approximate 20° angle. Layer top of gel with 1x TBE buffer to prevent drying of wells, and thoroughly wrap top of gel apparatus with saran wrap to prevent evaporation of buffer.

G. Preparation of PCR Products for Gel Analysis

1. Add 12.5 µl formamide loading buffer to each sample.

2. Prepare DNA marker by mixing 1 µl of fluorescein-labeled phi x174 cut with HinfI (previously prepared; see below), 10 µl 1x TBE, and 5.5 µl formamide loading buffer in a 200µl PCR tube.

3. To denature DNA, place samples into thermal cycler and start "boil" program (3 min 95°C, 4°C indefinitely) under the user option "Vicki".

H. Preparation of Sequencing Gel for Operation and Electrophoresis

1. Remove precision caster base injection port from gel apparatus (see manufacturer's instructions). Remove saran wrap, and slowly remove comb using a straight upward motion.

2. Set up gel apparatus according to manufacturer's instructions.

   a. Fill universal base with approximately 400 ml 1x TBE. Remove any bubbles from underside of gel with sweeping streams of buffer, using syringe and needle.

   b. After inserting stabilizer bar into the universal base, fill top chamber of gel apparatus with 1x TBE to approximately 1.5 cm above wells. Remove any bubbles from wells with gentle streams of buffer, using syringe and needle (if wall of a well is distorted through this process, straighten wall with a sequencing tip).

   d. Gently rub lubricating jelly on suction cup of temperature probe (connected to power supply) and attach to middle of front glass plate.

3. Rinse wells with 1x TBE.

4. Turn on power supply, and set temperature to 50°C, power to 70 watts, and pre-run gel for 45 minutes.

5. Rinse wells with 1x TBE just prior to loading samples. Using sequencing pipette tips, load 6 µl of sample (DNA marker or PCR product) into each well. Samples should be loaded in duplicate and

can be loaded in regular room lighting, however, gels should be run in darkness to avoid over exposure of fluorescent materials.

5. Attach bottom and top safety covers to gel apparatus, and connect electrical cables to power supply. Program power supply to run at 50 °C, 70 watts for approximately 3-4 hours. The xylene–cyanol band of the loading buffer should be at the very bottom of the gel.

I. Removal of Sequencing Gel from Apparatus

1. Turn off power supply and remove safety covers.

2. Drain upper buffer chamber of gel apparatus by inserting drain port connector into drain port of the back glass plate.

3. Remove stabilizer bar and drain remaining buffer by tilting gel apparatus.

4. Remove lever clamps and orient glass plate assembly on a horizontal surface covered with diaper paper such that back glass plate is facing up and bottom of glass plate assembly is facing away.

5. Very slowly, lift back glass plate from front glass plate; the gel will remain adhered to front plate.

6. Using a clean razor blade, cut upper left corner of gel to mark position of lane 1. Also, measure the length of gel (from bottom of wells upward) that will fit size of fluoroimager glass tray; remove top portion of gel that does not fit with a razor blade.

7. Place fluoroimager glass tray into a tub filled with 2 liters distilled water .

8. Orient lane 1 of gel with "A,1" corner of fluoroimager glass tray (flip front glass plate/gel assembly 180 °, such that front glass plate is facing up and gel is facing down). Slowly lower front glass plate/gel assembly into water, and allow gel to roll off glass plate onto glass tray.

9. Carefully lift glass tray/gel assembly out of water without allowing gel to slide off glass tray, dry underside of glass tray with kimwipes, and wrap glass tray/gel assembly in foil to avoid exposure to light while transporting to fluoroimager (Molecular Dynamics no. SSI-120).

J. Fluoroimaging

1. Select "scanner control" and door "open". Remove foil from glass tray/gel assembly, dry underside of glass tray with kimwipes, and slide tray/gel into fluoroimager opening.

2. Select door "close", "filter 1", and then "setup".

3. Select filter 1 "530df30 for 1% agarose ", gel orientation "R", detection sensitivity "normal", dye "single label", pixel size "100 microns", and dig. res. "16 bits".

4. Make certain the 530df30 filter is inserted into top of fluoroimager, and then select "o.k.".

5. Starting with upper left-hand corner (A,1), drag pointer to far lower right-hand corner (J,13) so that square is highlighted as much as possible.

6. Save gel scan under e:\BRI-NET\users\LSC.grp\data, and name gel scan hmr**date**.gel (e.g. a gel scannned on 6/23/97 should be named hmr62397.gel).

7. Select "o.k.", and then "scan".

K. Densitometry

1. Select "ImageQuant" and retrieve gel scan under file menu and e directory.

2. Maximize field of view for image of gel scan, and adjust contrast (darkness of bands against background) by selecting the gray color adjust option under VIEW toolbar.

3. Draw a solid line through middle of bands in each lane by selecting the "line" icon.

4. From object menu, select "object manager" to delete and redraw any solid lines that are not straight, and select "object attributes" to adjust width value so that dotted lines flank outer edges of bands.

5. From analysis menu, select "peak finder". The default values of peak finder should be as follows: auto detect "checked", display and print "not checked", sensitivity "8", kernel "5", and baseline "automatic". Select "compute" and a graph of peaks, representing each lane and corresponding bands, will be displayed.

6. Adjust sensitivity and kernel values so that graphs display the least amount of distinct peaks. Select "compute" after each adjustment to view graphs.

7. When adjustment is complete, select "print" from file menu to print graphs. Select "print" in peak finder window, and then "compute", to print numerical values for all peaks.

8. Close graph and peak finder windows. Select "print" from file menu to print picture of gel scan with the solid lines. Print another picture of gel scan without solid lines by deleting lines as indicated in step 4. Select "enlargement" icon to enlarge bands, and reprint gel scan.

L. Ratio Quantitation of Polymorphic Alleles

1. Compute ratio between numerical value of peaks corresponding to the two predominant bands in each lane of gel.

   Note: Allelic polymorphism of the human androgen receptor gene results from a difference in number of trinucleotide repeats at the particular locus flanked by HUMARA primers previously described (Busque et al.); therefore, DNA fragments differing in length are amplified via PCR. The PCR products are separated by size using gel electrophoresis, and are later visualized as distinct

bands. Observation of only one predominant band indicates a lack of polymorphism; in this case, a ratio cannot be determined.

2. PCR should be repeated in order to assess accuracy and reproducibility of ratios obtained.

Preparation of Fluorescein-Labeled Phi X174/Hinf Marker:

1. Prepare following mixture in a 1.5ml microcentrifuge tube wrapped with foil:

   5 µl 10x Klenow fill-in buffer,
   2 µl dATP (10mM)
   2 µl dCTP (10mM)
   2 µl dGTP (10mM)
   10 µl fluor-12-dUTP (1mM)
   6 µl phi x174/HinfI DNA marker (500µg/ml)
   4 µl Klenow polymerase (5U/µl)
   19 µl ddH_20
   final volume =50 µl

2. Incubate reaction mixture for 15 min at room temperature, followed by 5 min at 70°C.

3. To precipitate DNA, add 17ul of 10M NH_4OAc and 167ul of 100% ethanol. Incubate for 1 hour at -70°C.

4. To pellet DNA, centrifuge for 15 min at 4°C and 14000rpm in a microcentrifuge.

5. Aspirate supernatant. Wash pellet by adding 500ul of ice-cold 70% ethanol (DO NOT resuspend pellet), and centrifuge for 5 min as stated in step 4. Repeat wash 1x.

6. Cover opening of microcentrifuge tube with a double-folded piece of parafilm, and puncture several holes through the parafilm using a neddle or toothpick.

7. Remove any traces of ethanol by vacuum drying pellet for 5-10 min in a speed vac.

8. Resuspend pellet in 50 µl of TE pH 8.0.

9. Repeat steps 3-7 (for removal of unincorporated nucleotides).

10. Resuspend pellet in 20 µl of TE pH 8.0. Store flourescein-labeled DNA marker at -20°C. Keep away from light at all times.

Subject: HUMARA Assay

Reagents:

PBS, 1x (Irvine Scientific no. 9240). Store at 4°C. Expiration date per manufacturer.

Histo-Paque-1077 (Sigma no. H 1077-1). Store at 4°C. Expiration date per manufacturer.

RNase A, 20mg/ml. Store at -20°C. Expiration date per manufacturer.
1 ml Sterile water
20 mg RNase A (Boehringer Mannheim no.109169, stored at -20°C)

Dynabeads® M 450 PAN-T (CD-2) (Dynal no. 111.01) Store at 4°C. Expiration date per manufacturer.

Fetal Bovine Serum (Sigma no. F-4135) Store at -20°C. Expiration date per manufacturer.

Triton X-100 (Sigma no.T-9284) Store at room temperature. No expiration date.
Working Solution:
0.32 M Sucrose (Mallinkrodt Chemical no.8360)
10 mM Tris pH 7.5
5 mM MgCl2 (Mallinkrodt Chemical no. 5958)
1% Triton X-100
Autoclave

Aquaphenol (Oncor no.130181). Store at +4°C. Expiration date 6 months

Chloroform (Mallinkrodt Chemical no. 1888). Store at room temperature. Expiration date per manufacturer.

RPMI (Irvine Scientific no.9160). Store at +4°C. Expiration date per manufacturer.

NaCl (Sigma no. S-9625). Store at room temperature. Expiration date: none.

Genomic DNA Isolation Kit (QIAGEN no. 29104).

Proteinase K, 20mg/ml. Store at 4°C. Expiration date per manufacturer.
1.4 ml Sterile water
25 mg Proteinase K (QIAGEN no. 1000344, stored at 4°C)

Buffer AL. Store at room temperature. Expiration date per manufacturer.
12 ml Reagent AL1 (QIAGEN no. 1001489, stored at room temperature)
3 ml Reagent AL2 (QIAGEN no. 1001491, stored at room temperature)

Buffer AW. Store at room temperature. Expiration date per manufacturer.
40 ml Ethyl alcohol, 100% (Millennium Petrochemicals, stored at room temperature)
17 ml Reagent AW (QIAGEN no. 1003568, stored at room temperature)

Ethyl Alcohol, 100% (Millennium Petrochemicals 1gal). Store at room temperature.
Expiration date: none.

L Buffer, 10x (Boehringer Mannheim no.1417975). Store at -20°C. Expiration date per manufacturer.

Subject: HUMARA Assay

HpaII Restriction Enzyme, 40U/ul (Boehringer Mannheim no. 1207598). Store at -20°C. Expiration date per manufacturer.

RsaI Restriction Enzyme, 40U/ul (Boehringer Mannheim no. 1047671). Store at -20°C. Expiration date per manufacturer.

SIGMA Taq DNA Polymerase Kit. All components should be stored at -20°C. Expiration date per manufacturer.

SIGMA Taq DNA Polymerase, 5U/ul (Product # D4545)
10x PCR Buffer II (Product # P 2317)
$MgCl_2$, 25mM. (Product # M 8787)

Dimethyl Sulfoxide (SIGMA no. D-5879). Store at room temperature. Expiration date per manufacturer.

dNTP Cocktail, 2.5mM each. (COH Cloning Lab - Dr. Gerald Forest or Judy Ramos @ x:3322). Store at -20°C. Expiration date per manufacturer.

90.0 ul	Sterile water
2.5 ul	dATP, 100mM (Boehringer Mannheim no. 1051440, stored at -20°C)
2.5 ul	dCTP, 100mM (Boehringer Mannheim no. 1051458, stored at -20°C)
2.5 ul	dGTP, 100mM (Boehringer Mannheim no. 1051466, stored at -20°C)
2.5 ul	dTTP, 100mM (Boehringer Mannheim no. 1051482, stored at -20°C)

Fluorescein-Labeled HUMARA Primer I, 150pM. (COH Peptide Synthesis Facility - Dr. Piotr Swiderski or Peter Walker @ x:8372). Store at -20°C.
5¹-GCT GTG AAG GTT GCT GTT CCT CAT-3¹
HUMARA Primer II, 150pM. (COH Peptide Synthesis Facility - Dr. Piotr Swiderski or Peter Walker @ x:8372). Store at -20°C.
5¹-TCC AGA ATC TGT TCC AGA GCG TGC-3¹

HUMARA Primer III 150pM. (COH Peptide Synthesis Facility - Dr. Piotr Swiderski or Peter Walker @ x:8372). Store at -20°C.
5¹-GTT AGG GCT GGG AAG GGT CT-3¹
HUMARA Primer IV 150 pM. (COH Peptide Synthesis Facility - Dr. Piotr Swiderski or Peter Walker @ x:8372). Store at -20°C.
5¹-TCT GGG ACG CAA CCT CTC TC-3¹

Sigmacote (SIGMA no. SL-2). Store at 4°C. Expiration date per manufacturer.

Sequagel-4/Sequagel-6 Gel Solution (National Diagnostics no. EC-836). Store at room temperature. Expiration date per manufacturer.

[4 or 6 % Acrylamide (19:1; acrylamide/bisacrylamide), 6 M Urea pH 8.3 in 1x TBE]

Sequagel-Complete Buffer Reagent (National Diagnistics no. EC-841). Store at room temperature. Expiration date per manufacturer.

Subject: HUMARA Assay

Ammonium Persulfate (SIGMA no. A-1433, stored at room temperature) Store at room temperature.

NaOH, 10N. Store at room temperature. Expiration date: none.

80 ml	Sterile water
40 g	Sodium hydroxide (Mallinckrodt no. 7708-500, stored at room temperature) volume to Sterile water 100 ml

EDTA, 0.5M pH 8.0. Store at room temperature. Expiration date: none.

80 ml	Sterile water
18.6g	EDTA disodium salt-dihydrate (SIGMA no. ED2SS, stored at room temperature)
pH to 8.0	NaOH, 10N (see above, stored at room temperature)
volume to 100 ml	Sterile water

TBE, 5x. Store at room temperature. Expiration date: none (dispose if precipitate develops).

54 g	Tris base (Boehringer Mannheim no. 604203, stored at room temperature)
27.5g	Boric acid (J.T. Baker no. 0084-01, stored at room temperature)
20 ml	EDTA, 0.5M pH8.0 (see above, stored at room temperature)
volume to 1000 ml	Sterile water

Formamide Loading Buffer. Store at -20°C. Expiration date: none.

4.75 ml	Formamide (SIGMA no. F-4761, stored at 4°C)
200 ul	EDTA, 0.5M pH8.0 (see above, stored at room temperature)
2.5 mg	Bromophenol blue (SIGMA no. B-8026, stored at room temperature)
2.5 mg	Xylene cyanol (SIGMA no. X-4126, stored at room temper
50 ul	Sterile water

Klenow Fill-In Kit (Stratagene no. 200410).

Klenow Fragment of DNA Polymerase, 5U/ul. Store at -20°C. Expiration date per manufacturer.

Klenow Fill-In Buffer, 10x. Store at -20°C. Expiration date per manufacturer.

dATP, 10mM. Store at -20°C. Expiration date per manufacturer.

dCTP, 10mM. Store at -20°C. Expiration date per manufacturer.

dTTP, 10mM. Store at -20°C. Expiration date per manufacturer.

Fluor-12-dUTP, 1mM (Stratagene no. 300383). Store at -20°C. Expiration date per manufacturer.

Phi X174 HinfI DNA Fragments, 0.5mg/ml (Stratagene no. 201102). Store at -20°C. Expiration date per manufacturer.

NH_4OAc, 10M. Store at room temperature. Expiration date: none.

80 ml	Sterile water
77 g	Ammonium acetate (SIGMA no. A-8920, stored at 4°C)
volume to 100 ml	Sterile water

Tris, 1M pH 7.5. Store at room temperature. Expiration date: none.

80 ml	Sterile water
12.1g	Tris base (Boehringer Mannheim no. 604203, stored at room temperature)
pH to 7.5	
volume to 100 ml	Sterile water

TE, pH8.0. Store at room temperature. Expiration date: none.

494 ml	Sterile water
5 ml	Tris, 1M pH8.0 (see above, stored at room temperature)
1 ml	EDTA, 0.5M pH8.0 (see above, stored at room temperature)

References:

Busque et al. (1996). Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. Blood 88, 59-65.

Edwards et al. (1992). Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12, 241-253.

Legare et al. (1996). Analysis of clonality in therapy-related acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS) after autologous bone marrow transplant (ABMT) for lymphoma. Submitted for journal publication in Blood; not published.

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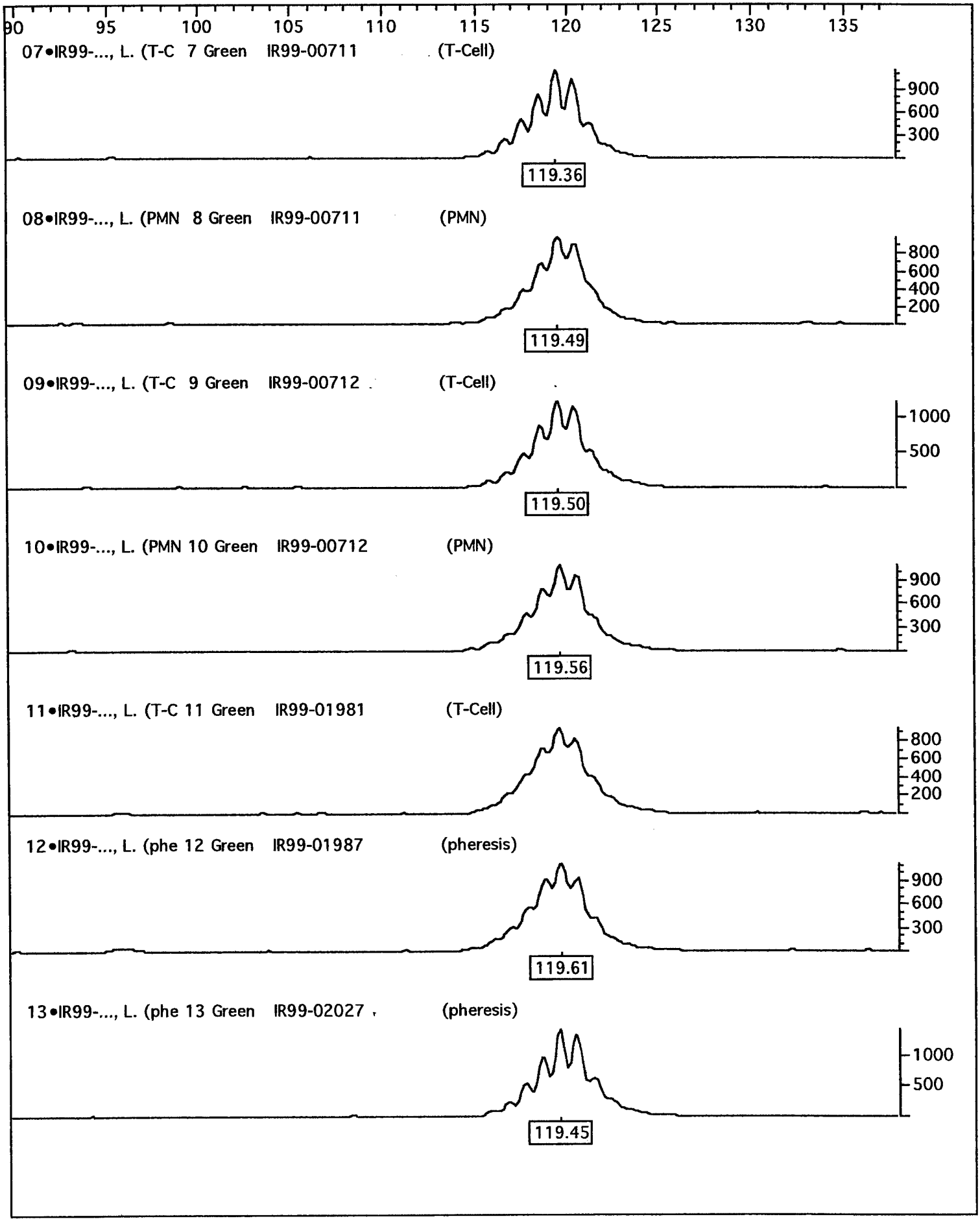
Reviewed and Edited by: Joyce Murata Collins, Ph.D./ Marilyn L. Slovak, Ph.D.

EXHIBIT 6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800 600 400 200
119.49
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119.50
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900 600 300
119.56
11•IR99-..., L. (T-C 11 Green IR99-01981 (T-Cell)
800 600 400 200
12•IR99-..., L. (phe 12 Green IR99-01987 (pheresis)
900 600 300
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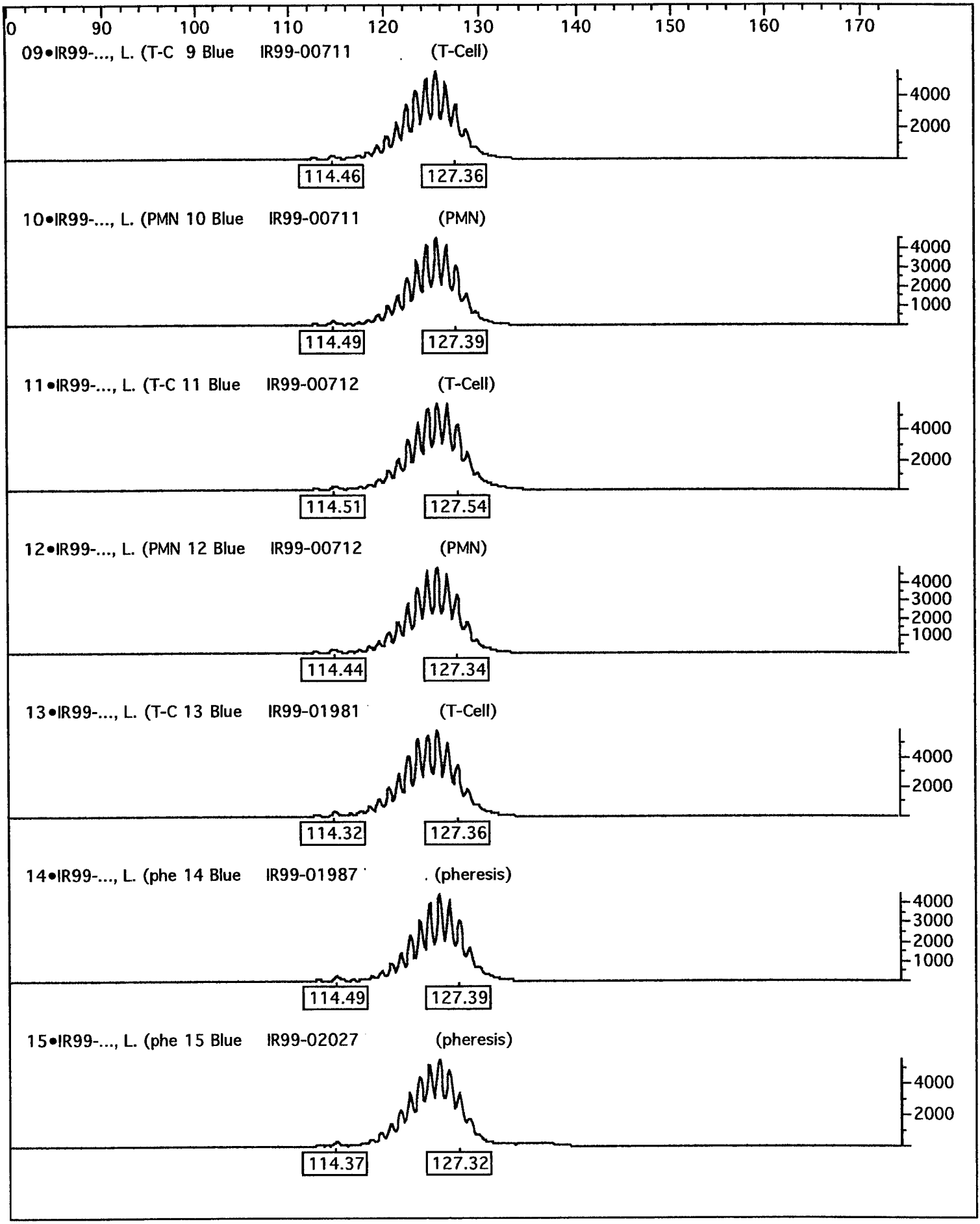
1000 500
119.45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127.54
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3000
2000
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114.44
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4000
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127.36
14•IR99-..., L. (phe 14 Blue IR99-01987 (pheresis)
4000
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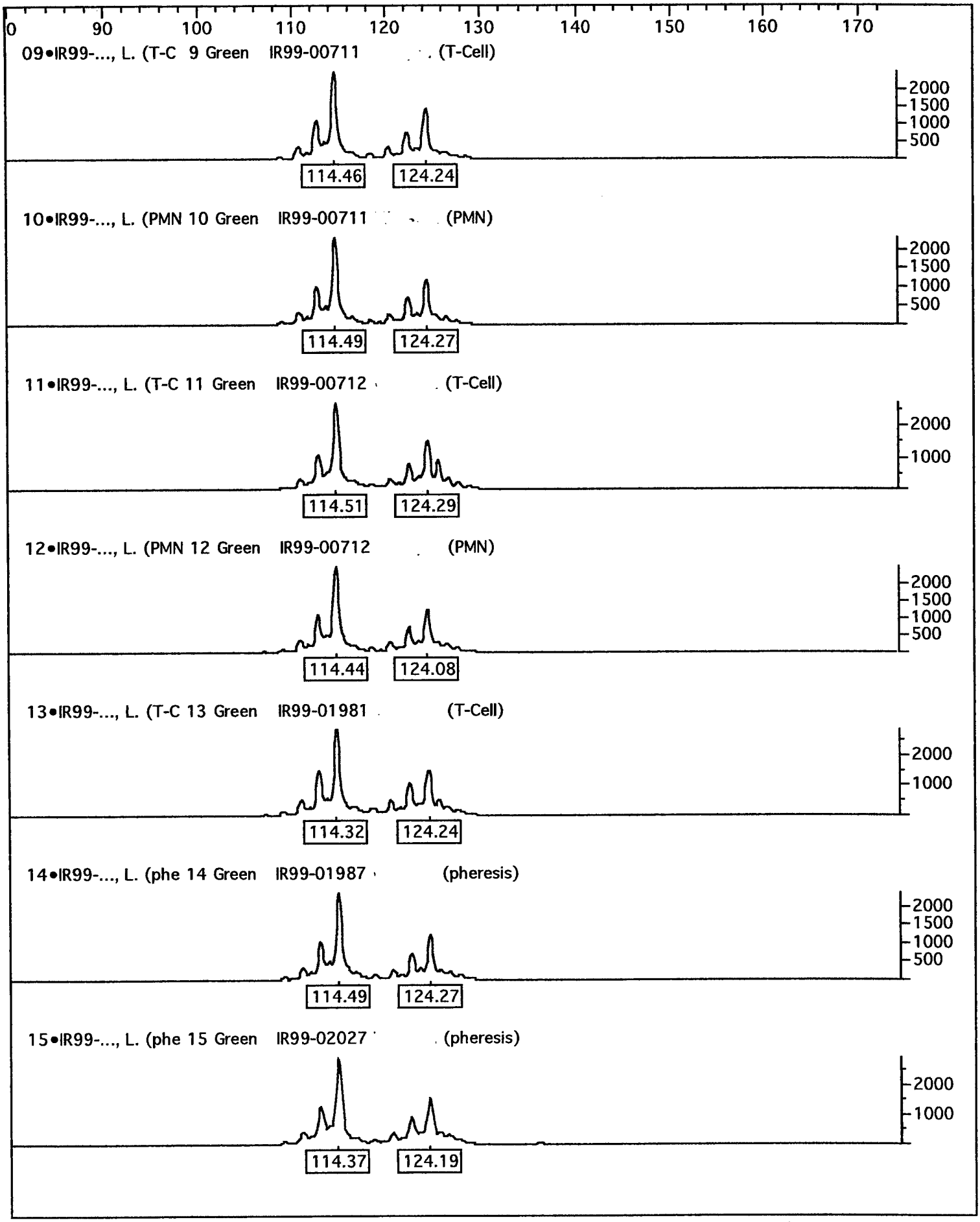
2000
114.37
127.32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2000 1500 1000 500
114.44 124.08
13•IR99-..., L. (T-C 13 Green IR99-01981 . (T-Cell)
2000 1000
114.32 124.24
14•IR99-..., L. (phe 14 Green IR99-01987 . (pheresis)
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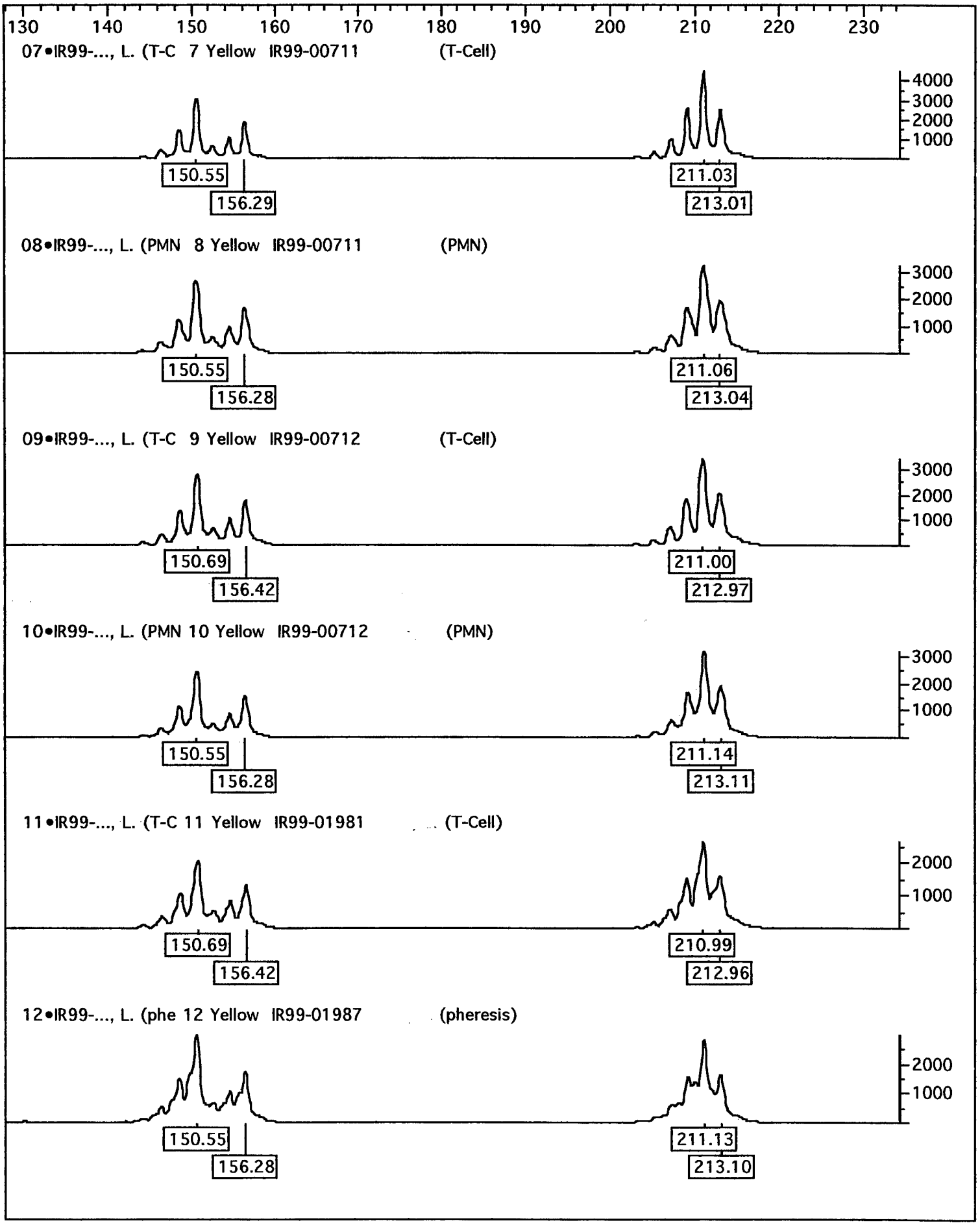
2000 1000
114.37 124.19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4000 3000 2000 1000
150.55 156.29 211.03 213.01
08•IR99-..., L. (PMN 8 Yellow IR99-00711 (PMN)
3000 2000 1000
150.55 156.28 211.06 213.04
09•IR99-..., L. (T-C 9 Yellow IR99-00712 (T-Cell)
3000 2000 1000
150.69 156.42 211.00 212.97
10•IR99-..., L. (PMN 10 Yellow IR99-00712 (PMN)
3000 2000 1000
150.55 156.28 211.14 213.11
11•IR99-..., L. (T-C 11 Yellow IR99-01981 (T-Cell)
2000 1000
150.69 156.42 210.99 212.96
12•IR99-..., L. (phe 12 Yellow IR99-01987 (pheresis)
2000 1000
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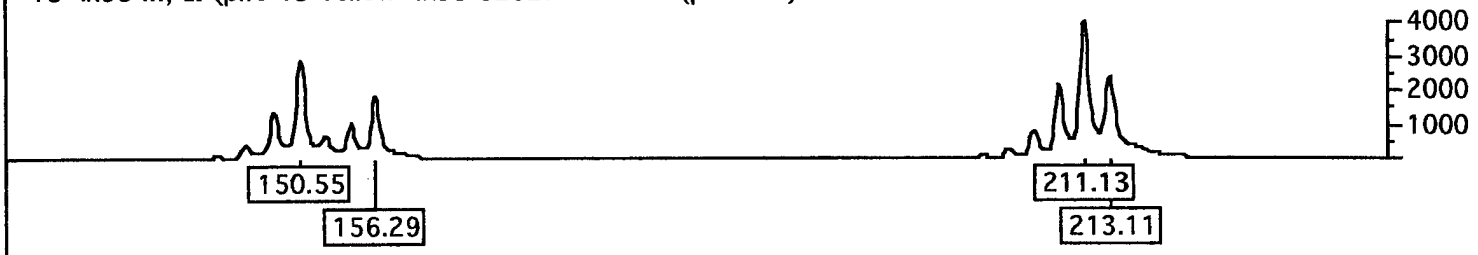
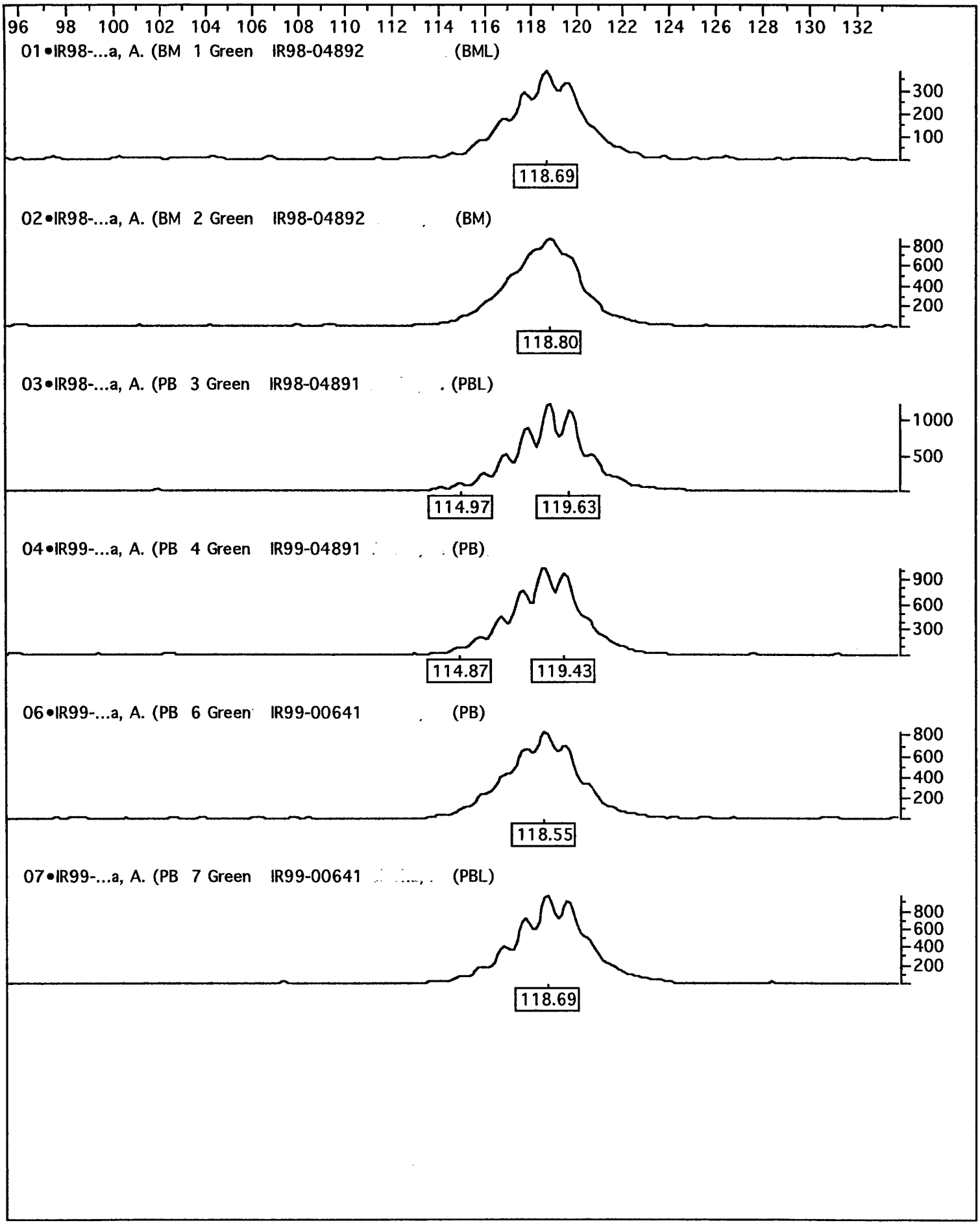
13•IR99-..., L. (phe 13 Yellow IR99-02027 - (pheresis)

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200
100
118.69
02•IR98-...a, A. (BM 2 Green IR98-04892 (BM)
800
600
400
200
118.80
03•IR98-...a, A. (PB 3 Green IR98-04891 (PBL)
1000
500
114.97
119.63
04•IR99-...a, A. (PB 4 Green IR99-04891 (PB)
900
600
300
114.87
119.43
06•IR99-...a, A. (PB 6 Green IR99-00641 (PB)
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600
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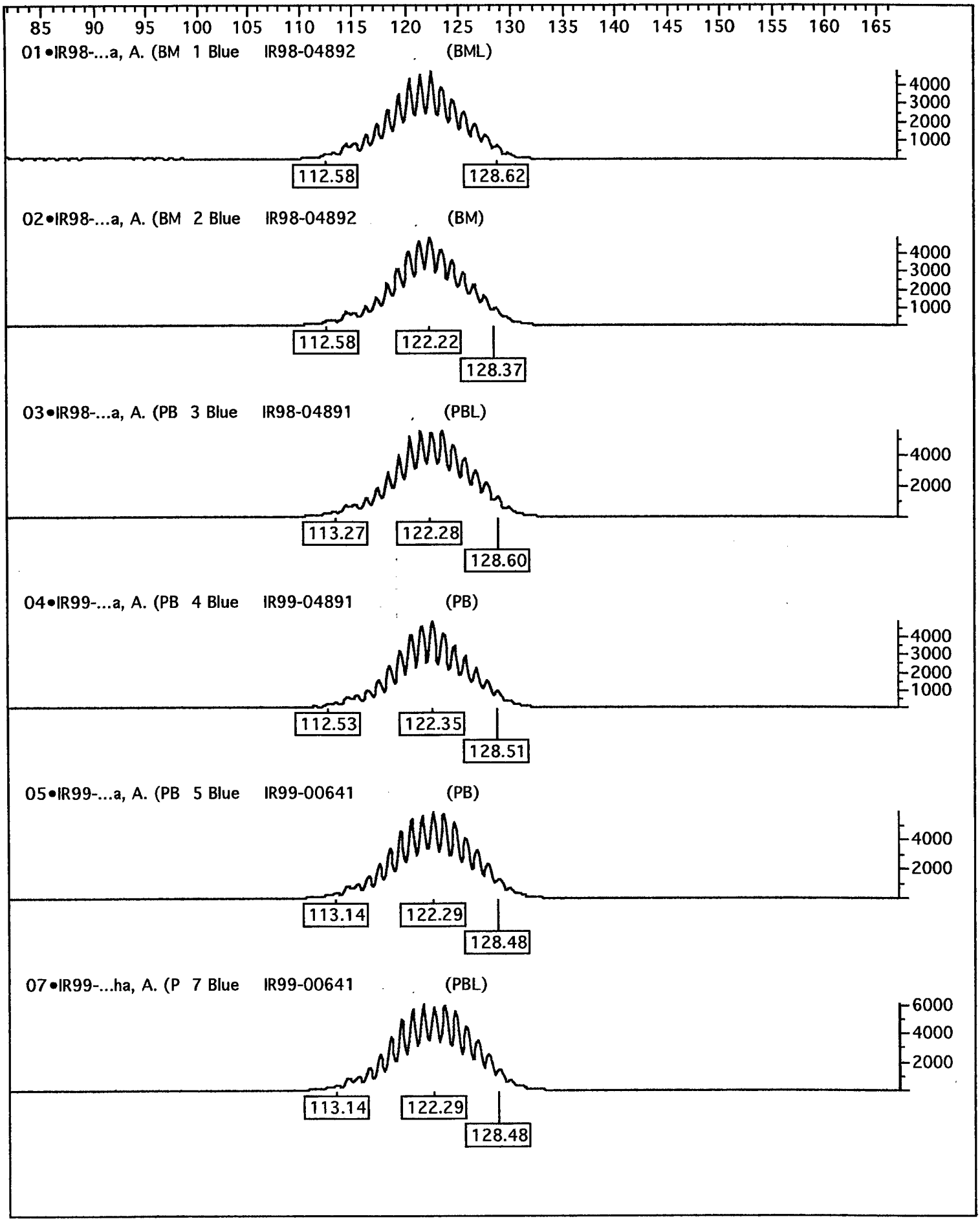
200
118.55
07•IR99-...a, A. (PB 7 Green IR99-00641 (PBL)
800
600
400
200
118.69


85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165
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4000
3000
2000
1000
112.58
128.62
02•IR98-...a, A. (BM 2 Blue IR98-04892 (BM)
4000
3000
2000
1000
112.58
122.22
128.37
03•IR98-...a, A. (PB 3 Blue IR98-04891 (PBL)
4000
2000
113.27
122.28
128.60
04•IR99-...a, A. (PB 4 Blue IR99-04891 (PB)
4000
3000
2000
1000
112.53
122.35
128.51
05•IR99-...a, A. (PB 5 Blue IR99-00641 (PB)
4000
2000
113.14
122.29
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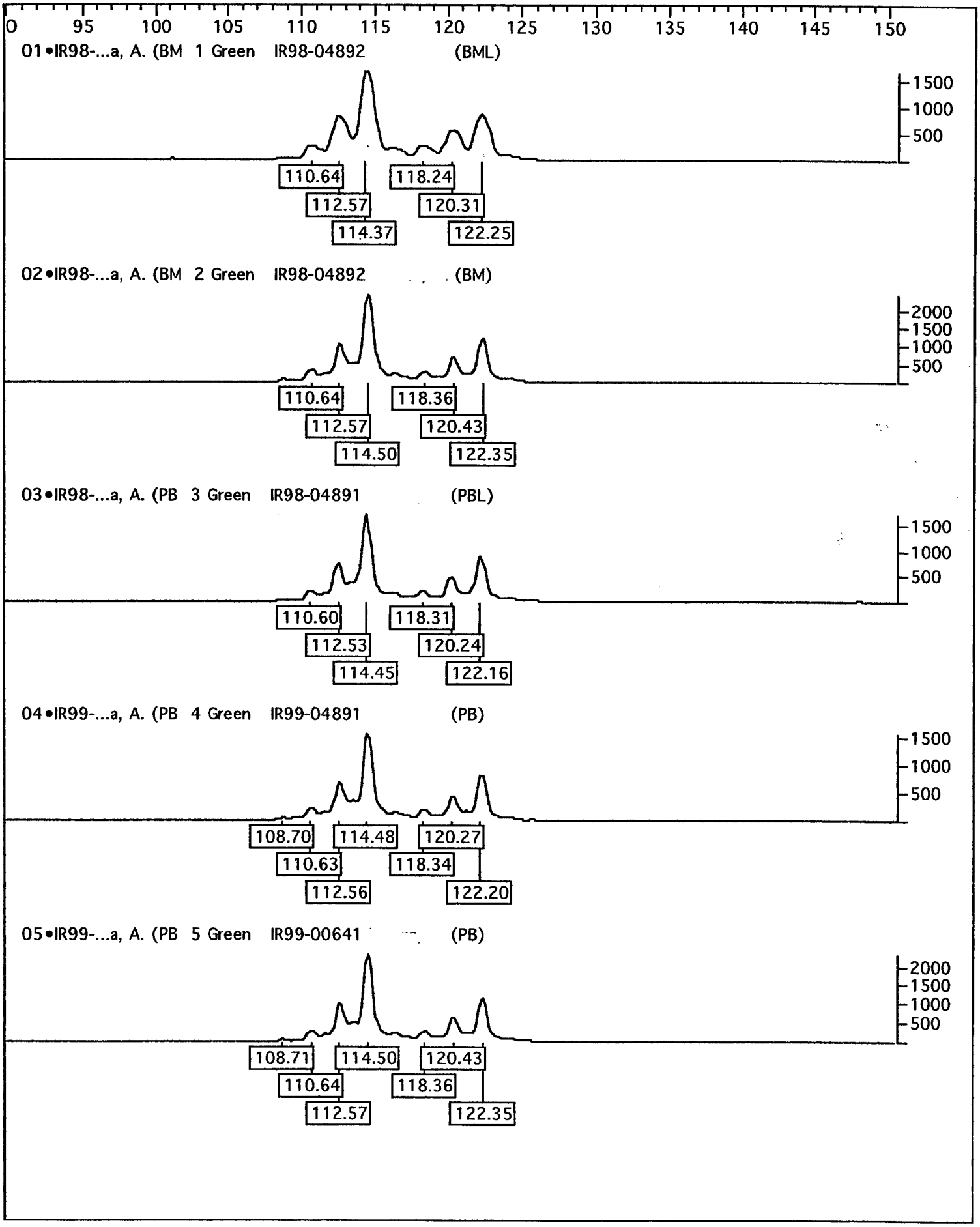
07•IR99-...ha, A. (P 7 Blue IR99-00641 (PBL)
6000
4000
2000
113.14
122.29
128.48


0 95 100 105 110 115 120 125 130 135 140 145 150
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1000
500
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112.57
114.37
118.24
120.31
122.25
02•IR98-...a, A. (BM 2 Green IR98-04892 (BM)
2000
1500
1000
500
110.64
112.57
114.50
118.36
120.43
122.35
03•IR98-...a, A. (PB 3 Green IR98-04891 (PBL)
1500
1000
500
110.60
112.53
114.45
118.31
120.24
122.16
04•IR99-...a, A. (PB 4 Green IR99-04891 (PB)
1500
1000
500
108.70
110.63
112.56
114.48
118.34
120.27
122.20
05•IR99-...a, A. (PB 5 Green IR99-00641 (PB)
2000
1500
1000
500
108.71
110.64
112.57
114.50
118.36
120.43
122.35

06•IR99-...ha, A. (P 6 Green IR99-00641 (PBL)